(54) Title: METHOD FOR THE PRODUCTION OF RECOMBINANT VIRUS, DNA CONSTRUCTS, RECOMBINANT VIRUS AND VACCINE COMPOSITIONS
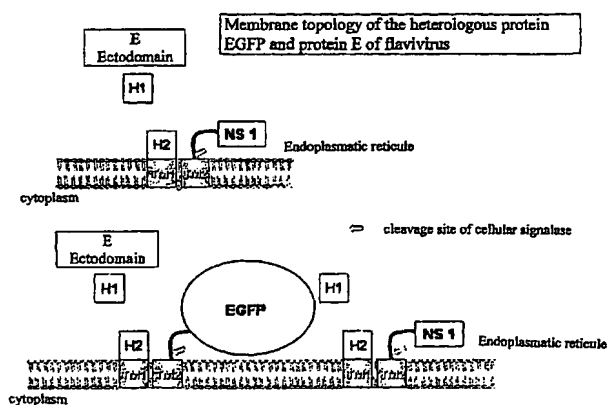
(57) Abstract: The purpose of the present invention is the production of recombinant virus through the cloning and expression of sequences of coding nucleotides of the whole or part of heterolog proteins, through the following method: (a) modification of the heterolog nucleotides sequences in such way they when cloned and expressed in the vector virus, they present in the 5' region, nucleotides present in the 5' edge of the gene NS1 of this vector virus or of other virus or equivalent functional sequences, and in its 3' region, the correspondent genome region in the whole or part of the spheres of the steam and anchor of the protein E of this vector virus or equivalent functional sequences, and not compromising the structure and the replication of the mention vector virus; (b) insertion of the modified heterolog sequences in (a) in the intergene region at the structural protein E level and of onstructural NS1 vector virus; (c) obtention of the non pathogenic recombinant virus and owner of the immunologic properties, having the heterolog sequences integrated in the viral genome according to the insertion described in (b) and, like that, expressing the heterolog antigene in such way that it can induce an appropriate immune response. The present invention is also addressed to vaccine compositions to immune against the Flavivirus and/or other patogens.

METHOD FOR THE PRODUCTION OF RECOMBINANT VIRUS, DNA CONSTRUCTS, RECOMBINANT VIRUS AND VACCINE COMPOSITIONS

The present invention is related to the genetic manipulation of virus, including, but not limited to,

5   Flavivirus, mainly the vaccine amarilico virus 17D strain or its derivatives; resulting in recombinant virus containing heterolog nucleotides coming from from other pathogens among the genes which codify the viral proteins E and NS1. Such recombinant virus, resulting from its

10 attenuation characteristics, imunogeneticity and genetic stability, may be applied in the development of attenuated alive vaccines to human and animal use, granting immune response not only to the Yellow Fever or any other disease caused by virus, but also to diseases caused by other

15 pathogens.

Background Of Invention

The *Flaviviridae* family includes three genera: Flavivirus, having as main representatives the virus of the yellow fever, the virus of dengue, the virus of the

20 Japanese encephalite; the genera Hepacivirus (virus of hepatite C) and the genera of Pestivirus (virus of diarrhea bovine). Eventhough they belong to different genera, with distinct biological properties and without crossed sorological reactivity, the virus of the 3 types share a

25 great similarity in the viral morphology, in the genomic organization and in the replication strategy (Rice, C. M. 1996. Flaviviridae: the viruses and their replication, Third ed, vol. 1. Lippincott-Raven, Philadelphia, PA).

The virus of the yellow fever is the prototype of the genera <u>Flavivirus</u> from the family *Flaviviridae*, which includes about 70 virus. The flavivirus are small (40-60 nm), spherical, enclosed, with RNA genome of single strain, with the majority of these arbovirus called as such due to their transmission by arthropod-born viruses ("arthropod-borne viruses"), as mosquitos or ticks, causing important diseases on man and animals.

Figure 1 presents the genomic organization of the Flavivirus (Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice. 1990. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44:649-88). The genome is represented on the top part, with the indication of the 5' and 3' non translated sequences and the open reading phase of 10.862 nucleotides. On this reading phase, 5'→ 3' direction, the three structural proteins (C, prM and E) and the seven genes to the non structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5) are codified. The arrows indicated the proteolitic clivage sites performed by the viral protease (NS2B/NS3); and the lozenges, the cleavages by the cellular signalase (occurs inside the endoplasmatic reticule). The asterisks indicate the glicosilation sites linked to asparagines.

The yellow fever virus (Figure 1) has a genome constituted by one single RNA molecule with 10.862 nucleotides (nt), one CAP structure at the 5' edge(^{m7}GpppG, to be recognized by the ribossomes), 5' region non translated short (118 nt) and a 3' edge not poliadenilated(511 nt). Such data were obtained from the

first nucleotide sequencing of flavivirus genome - the

vaccine virus vacinal 17D-204 (Rice, C. M., E. M. Lenches,

S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss.

1985. Nucleotide sequence of yellow fever virus:

5   implications for flavivirus gene expression and evolution.

Science 229:726-33).

In the cytoplasm of the host cell, the viral RNA is

used as a shape to the synthesis of the negative

complementary strain, which, by its turn, will be the shape

10   to the synthesis of more positive strains to be used in the

set up of new viral particles. The replication is a semi

conservative   process   and   involves   replicative

intermediates, as well as replicative ways. The formation

of viral particles occurs through the relationship of the

15   viral nucleocapsid, with the envelope protein anchored on

the membrane of the cellular Endoplasmatic Reticule (RER).

The set up of viral particles occurs in very close

association with the RER. The viral particles are carried

through vesicles and, from that point, released by the

20   exocytose through the Golgy system.

The RNA is also the viral messenger and the

transduction of infected cells results in the synthesis of

a poliprotein forerunner of 3.411 aminoacids, which, when

proteolitically   processed,   create   the   10   viral

25   polypeptides. From the 5' edge, the order of genes is C;

prM/M; E; NS1; NS2A; NS2B; NS3; NS4A; NS4B and NS5. The

three first genes codify the structural viral proteins,

that means, the ones which form the virus together with the

encapsid RNA molecule, being denominated as capsid (C, 12-

14kDa), membrane (M of 8kDa, and its forerunner prM of 18-22 kDa) and envelope (E, 52-54 kDa). These three genes are transcoded in the first quarter of the genome. The remaining genome codifies the non structural proteins (NS),

5   numbered from 1 to 5 (NS1 a NS5), in accordance with the order of synthesis (Rice, C. M., E. M. Lenches, S. R. Eddy, S. J. Shin, R. L. Sheets, and J. H. Strauss. 1985. Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229:726-

10   33).

Among the different Flavivirus, three great non structural proteins have very well conserved sequences: NS1 (38-41 kDa), NS3 (68-70 kDa) and NS5 (100-103 kDa).

The first one (NS1) has an important role in the

15   replication of the negative strand of RNA (Lindenbach, B. D., and C. M. Rice. 1999. Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function. J Virol 73:4611-21; Lindenbach, B. D., and C. M. Rice. 1997. trans-Complementation of yellow fever

20   virus NS1 reveals a role in early RNA replication. J Virol 71:9608-17; Muylaert, I. R., T. J. Chambers, R. Galler, and C. M. Rice. 1996. Mutagenesis of the N-linked glycosylation sites of the yellow fever virus NS1 protein: effects on virus replication and mouse neurovirulence. Virology

25   222:159-68; Muylaert, I. R., R. Galler, and C. M. Rice. 1997. Genetic analysis of the yellow fever virus NS1 protein: identification of a temperature-sensitive mutation which blocks RNA accumulation. J Virol 71:291-8). Released extracellularly as hexameric structure, may be located in

the cellular surface. Antibodies against NS1 do not

neutralize the viral infectivity, but exert protective

immunity through mediation of the complement lyzing

infected cells (Rice, C. M. 1996. Flaviviridae: the viruses

5   and their replication., Third ed, vol. 1. Lippincott-Raven,

Philadelphia, PA).

The second one, NS3, make up three distinct enzymatic

activities: (1) protease, being responsible for the

proteolytic process of the viral poliprotein in sites where

10   the cellular protease does not act (Lee, E., C. E. Stocks,

S. M. Amberg, C. M. Rice, and M. Lobigs. 2000. Mutagenesis

of the signal sequence of yellow fever virus prM protein:

enhancement of signalase cleavage In vitro is lethal for

virus production. J Virol 74:24-32; Stocks, C. E., and M.

15   Lobigs. 1995. Posttranslational signal peptidase cleavage

at the flavivirus C-prM junction in vitro. J Virol 69:8123-

6; Yamshchikov, V. F., and R. W. Compans. 1995. Formation

of the flavivirus envelope: role of the viral NS2B-NS3

protease. J Virol 69:1995-2003; Yamshchikov, V. F., D. W.

20   Trent, and R. W. Compans. 1997. Upregulation of signalase

processing and induction of prM-E secretion by the

flavivirus NS2B-NS3 protease: roles of protease components.

J Virol 71:4364-71); (2) helicase and (3) nucleotide-

trifosfatase (Gorbalenya, A. E., E. V. Koonin, A. P.

25   Donchenko, and V. M. Blinov. 1989. Two related

superfamilies of putative helicases involved in

replication, recombination, repair and expression of DNA

and RNA genomes. Nucleic Acids Res 17:4713-30; Wengler, G.,

and G. Wengler. 1993. The NS 3 nonstructural protein of

flaviviruses contains an RNA triphosphatase activity.
Virology 197:265-73; Wu, J., A. K. Bera, R. J. Kuhn, and J.
L. Smith. 2005. Structure of the Flavivirus helicase:
implications for catalytic activity, protein interactions,
5 and proteolytic processing. J Virol 79:10268-77). The two
last ones give to this protein an important role also in
the replication of the viral RNA.

The third one, NS5, is the greatest and most conserved
viral protein, making up the viral RNA polimerase, since
10 its sequence contains several structural elements
characteristic of RNA polymerases (Chambers, T. J., C. S.
Hahn, R. Galler, and C. M. Rice. 1990. Flavivirus genome
organization, expression, and replication. Annu Rev
Microbiol 44:649-88) and still exhibits RNA polimerase
15 activity, dependent of RNA (Steffens, S., H. J. Thiel, and
S. E. Behrens. 1999. The RNA-dependent RNA polymerases of
different members of the family Flaviviridae exhibit
similar properties in vitro. J Gen Virol 80 (Pt 10):2583-
90).

20   The four small proteins NS2A, NS2B, NS4A and NS4B are
not enough conserved in its aminoacid sequence, but not in
its patterns of multiple hydrophobic parts. These small
proteins were related, up to the moment, to some processes
of viral propagation: NS2A seems to be necessary to the
25 correct processing of NS1 (Falgout, B., R. Chanock, and C.
J. Lai. 1989. Proper processing of dengue virus
nonstructural glycoprotein NS1 requires the N-terminal
hydrophobic signal sequence and the downstream
nonstructural protein NS2a. J Virol 63:1852-60) and to the

set up of the viral particle together with NS3 (Kummerer,
B. M., and C. M. Rice. 2002. Mutations in the yellow fever
virus nonstructural protein NS2A selectively block
production of infectious particles. J Virol 76:4773-84);

5   NS2B is associated with NS3, acting as a complex
proteolitic viral cofactor (Chambers, T. J., A.
Nestorowicz, S. M. Amberg, and C. M. Rice. 1993.
Mutagenesis of the yellow fever virus NS2B protein: effects
on proteolytic processing, NS2B-NS3 complex formation, and

10 viral replication. J Virol 67:6797-807; Falgout, B., M.
Pethel, Y. M. Zhang, and C. J. Lai. 1991. Both
nonstructural proteins NS2B and NS3 are required for the
proteolytic processing of dengue virus nonstructural
proteins. J Virol 65:2467-75; Jan, L. R., C. S. Yang, D. W.

15 Trent, B. Falgout, and C. J. Lai. 1995. Processing of non-
structural Japanese encephalitis virus proteins: NS2B-NS3
complex and heterologous proteases. J Gen Virol 76 (Pt
3):573-80); NS4A would interact with NS1, allowing
itsintegration in the citoplasmatic process of RNA

20 replication (Lindenbach, B. D., and C. M. Rice. 1999.
Genetic interaction of flavivirus nonstructural proteins
NS1 and NS4A as a determinant of replicase function. J
Virol 73:4611-21). Considering that the synthesis of the
viral RNA occurs in the cellular cytoplasm in association

25 with membranes of RER, it is assumed that these viral
hydrophobic viral proteins would be immersed in membranes
and, through interactions with NS3 and NS5, they would be
participating with them in complex viral replicatives.

Structural elements present in the non translated 5'
and 3' edges (NTR) are also important in the replication and
wrapping of the viral RNA (Chambers, T. J., C. S. Hahn, R.
Galler, and C. M. Rice. 1990. Flavivirus genome
organization, expression, and replication. Annu Rev
Microbiol 44:649-88; Cologna, R., and R. Rico-Hesse. 2003.
American genotype structures decrease dengue virus output
from human monocytes and dendritic cells. J Virol 77:3929-
38; Elghonemy, S., W. G. Davis, and M. A. Brinton. 2005.
The majority of the nucleotides in the top loop of the
genomic 3' terminal stem loop structure are cis-acting in a
West Nile virus infectious clone. Virology 331:238-46;
Hanley, K. A., L. R. Manlucu, G. G. Manipon, C. T. Hanson,
S. S. Whitehead, B. R. Murphy, and J. E. Blaney, Jr. 2004.
Introduction of mutations into the non-structural genes or
3' untranslated region of an attenuated dengue virus type 4
vaccine candidate further decreases replication in rhesus
monkeys while retaining protective immunity. Vaccine
22:3440-8; Khromykh, A. A., H. Meka, K. J. Guyatt, and E.
G. Westaway. 2001. Essential role of cyclization sequences
in flavivirus RNA replication. J Virol 75:6719-28; Thurner,
C., C. Witwer, I. L. Hofacker, and P. F. Stadler. 2004.
Conserved RNA secondary structures in Flaviviridae genomes.
J Gen Virol 85:1113-24; Tilgner, M., T. S. Deas, and P. Y.
Shi. 2005. The flavivirus-conserved penta-nucleotide in the
3' stem-loop of the West Nile virus genome requires a
specific sequence and structure for RNA synthesis, but not
for viral translation. Virology 331:375-86; Tilgner, M.,
and P. Y. Shi. 2004. Structure and function of the 3'

terminal six nucleotides of the west nile virus genome in
viral replication. J Virol 78:8159-71; Yu, L., and L.
Markoff. 2005. The topology of bulges in the long stem of
the flavivirus 3' stem-loop is a major determinant of RNA
5   replication competence. J Virol 79:2309-24).

The protein C of the capsid interacts with the viral
RNA, forming the viral nucleocapsid (Chambers, T. J., C. S.
Hahn, R. Galler, and C. M. Rice. 1990. Flavivirus genome
organization, expression, and replication. Annu Rev
10 Microbiol 44:649-88). The protein prM is a glicosilated
forerunner of the membrane protein. It is present on the
surface of immature viral particles, with the cleavage by
cellular proteases *furina* type at the level of the Golgy
complex, before the release of viral particles, in such way
15 that the mature virus contains the protein M. The role of
the prM is to stabilize the protein E, avoiding the
premature show off of the fusion peptide to the reduced pH
found in the exocite via (Heinz, F. X., and S. L. Allison.
2003. Flavivirus structure and membrane fusion. Adv Virus
20 Res 59:63-97). The retention of prM protein may affect the
conformation and antigenicity of the protein E and reduce
the infectivity, inhibiting the acid-dependent fusion.

On Figure 2, the immature (intracellular form) and
mature (extracellular form) viral particles of the
25 Flavivirus are represented. The capsid of the virus has an
icosahedra symmetry, but the shape is not necessarily the
one presented on the Figure, which also shows the genome of
the virus associated with the internal side of the capsid.
Here are represented the envelope proteins (E) and its

dimeric form, the protein of the membrane (M) and its _forerunner_(prM), which is still present in the envelope in an extracellular shape. Oppositely to the extracellular particles, the intracellular particles are not infective

5   (Chambers, T. J., C. S. Hahn, R. Galler, and C. M. Rice. 1990. Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44:649-88).

The protein E is the main component of the viral envelope. It promotes the linkage to glicoproteic receptors

10   on the cellular surface and the internalization by dependent fusion of pH, processes that trigger a viral infection. This protein has multiple determinant antigens and it is the main target to the immune-protective response of the vertebrate host. Therefore, it plays a key role in

15   the cellular infections, in the viral tropism, in virulence and in the immunity.

The discovery of the three-dimensional atomic structure of the protein E of the mature viral particle of flavivirus TBE (tick-borne encephalitis virus), reveals

20   that this protein exists as a homodimers, about 110 kDa, with three defined spheres, anchored by the hydrophobic carboxylic edge on the envelope surface (Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison. 1995. The envelope glycoprotein from tick-borne encephalitis virus at

25   2 A resolution. Nature 375:291-8). This model has been seen applied to all Flavivirus, contributing mainly to the detection of antigen tracers and the study of mutations linked to the increase or decrease of virulence (Arroyo, J., F. Guirakhoo, S. Fenner, Z. X. Zhang, T. P. Monath, and

T. J. Chambers. 2001. Molecular basis for attenuation of neurovirulence of a yellow fever Virus/Japanese encephalitis virus chimera vaccine (ChimeriVax-JE). J Virol 75:934-42; Guirakhoo, F., Z. Zhang, G. Myers, B. W. Johnson, K. Pugachev, R. Nichols, N. Brown, I. Levenbook, K. Draper, S. Cyrek, J. Lang, C. Fournier, B. Barrere, S. Delagrave, and T. P. Monath. 2004. A single amino acid substitution in the envelope protein of chimeric yellow fever-dengue 1 vaccine virus reduces neurovirulence for suckling mice and viremia/viscerotropism for monkeys. J Virol 78:9998-10008; Halstead, S. B., F. X. Heinz, A. D. Barrett, and J. T. Roehrig. 2005. Dengue virus: molecular basis of cell entry and pathogenesis, 25-27 June 2003, Vienna, Austria. Vaccine 23:849-56; Hurrelbrink, R. J., and P. C. McMinn. 2003. Molecular determinants of virulence: the structural and functional basis for flavivirus attenuation. Adv Virus Res 60:1-42; Kolaskar, A. S., and U. Kulkarni-Kale. 1999. Prediction of three-dimensional structure and mapping of conformational epitopes of envelope glycoprotein of Japanese encephalitis virus. Virology 261:31-42; Lee, E., R. A. Hall, and M. Lobigs. 2004. Common E protein determinants for attenuation of glycosaminoglycan-binding variants of Japanese encephalitis and West Nile viruses. J Virol 78:8271-80; Lee, E., and M. Lobigs. 2000. Substitutions at the putative receptor-binding site of an encephalitic flavivirus alter virulence and host cell tropism and reveal a role for glycosaminoglycans in entry. J Virol 74:8867-75; Lee, E., C. E. Stocks, S. M. Amberg, C. M. Rice, and M. Lobigs.

2000. Mutagenesis of the signal sequence of yellow fever
virus prM protein: enhancement of signalase cleavage In
vitro is lethal for virus production. J Virol 74:24-32;
Mandl, C. W., S. L. Allison, H. Holzmann, T. Meixner, and
F. X. Heinz. 2000. Attenuation of tick-borne encephalitis
virus by structure-based site-specific mutagenesis of a
putative flavivirus receptor binding site. J Virol 74:9601-
9; Nickells, M., and T. J. Chambers. 2003. Neuroadapted
yellow fever virus 17D: determinants in the envelope
protein govern neuroinvasiveness for SCID mice. J Virol
77:12232-42; Ryman, K. D., H. Xie, T. N. Ledger, G. A.
Campbell, and A. D. Barrett. 1997. Antigenic variants of
yellow fever virus with an altered neurovirulence phenotype
in mice. Virology 230:376-80; Shirato, K., H. Miyoshi, A.
Goto, Y. Ako, T. Ueki, H. Kariwa, and I. Takashima. 2004.
Viral envelope protein glycosylation is a molecular
determinant of the neuroinvasiveness of the New York strain
of West Nile virus. J Gen Virol 85:3637-45).

The bonding of protein E to cell receptors leads to
the formation of de endocitic vesicles, covered by
clatrine. After the internalization by endocitose mediated
by receptor, the virus are released in the cytoplasm
through conformation changes, induced by acidic pH which
takes the peptide of fusion to be exposed after the
trimerization of protein E (Bonaldo, M. C., R. C. Garratt,
R. S. Marchevsky, E. S. Coutinho, A. V. Jabor, L. F.
Almeida, A. M. Yamamura, A. S. Duarte, P. J. Oliveira, J.
O. Lizeu, L. A. Camacho, M. S. Freire, and R. Galler. 2005.
Attenuation of recombinant yellow fever 17D viruses

expressing foreign protein epitopes at the surface. J Virol
79:8602-13; Bressanelli, S., K. Stiasny, S. L. Allison, E.
A. Stura, S. Duquerroy, J. Lescar, F. X. Heinz, and F. A.
Rey. 2004. Structure of a flavivirus envelope glycoprotein

5   in its low-pH-induced membrane fusion conformation. Embo J
23:728-38; Heinz, F. X., and S. L. Allison. 2003.
Flavivirus structure and membrane fusion. Adv Virus Res
59:63-97; Stiasny, K., S. Bressanelli, J. Lepault, F. A.
Rey, and F. X. Heinz. 2004. Characterization of a membrane-

10   associated trimeric low-pH-induced Form of the class II
viral fusion protein E from tick-borne encephalitis virus
and its crystallization. J Virol 78:3178-83).

In 1927, the virus which causes the yellow fever was
isolated in the Rhesus (*Macaca mulatta*), through the

15   straight inoculation of blood from an African patient named
Asibi (Stokes A, B. J., Hudson NP. 1928, The transmission
of yellow fever to Macacus rhesus. Rev Med Virol. 11:141-
148). After the set up of a pattern of an animal model
sensitive to the virus, new perspectives showed up and the

20   viral propagation and the clinical evaluation became
possible. The Asibi virus, the original sample, is one of
the most virulent among the yellow fever virus ever
studied. When inoculated in monkeys, through subcutaneous
via, in 4 to 7 days it caused death in 95% of the animals,

25   and high rates of viremia are detected in the blood of
theses infected animals.

The *serial* passage of Asibi cepa, in different types
of cultivation, as described priorly, lead to the
production of the parental 17D cepa, in the passage 180, to

17DD in the passage 195, and to 17D-204 cepa in the passage
204. The 17DD cepa was cultivated afterwards until the
passage 243 and suffered 43 extra passages in chicken
embryo (passage 286). The 17D-204 cepa, by its turn,

5   created by cultivation, to Colombia 88 cepa, that by its
turn, originated the different seed shares used in France
(I. Pasteur, passage 235) and in the United States
(Connaught, passage 234). The 17D-204 and 17DD virus are
the two sub cepas of the 17D cepas used actually to produce

10   vaccines in the world, which accumulated the genotype and
phenotype differences due to the independent serial
passages (Galler, R., P. R. Post, C. N. Santos, and
Ferreira, II. 1998. Genetic variability among yellow fever
virus 17D substrains. Vaccine 16:1024-8; Marchevsky, R. S.,

15   M. S. Freire, E. S. Coutinho, and R. Galler. 2003.
Neurovirulence of yellow fever 17DD vaccine virus to rhesus
monkeys. Virology 316:55-63; Post, P. R., R. de Carvalho,
M. da Silva Freire, and R. Galler. 2001. The early use of
yellow fever virus strain 17D for vaccine production in

20   Brazil--a review. Mem Inst Oswaldo Cruz 96:849-57).
However, both are equally immunogenic and safe for human
vaccine (Camacho, L. A., S. G. Aguiar, M. D. Freire, M. D.
Leal, J. P. Nascimento, T. Iguchi, J. A. Lozana, and R. H.
Farias. 2005. Reactogenicity of yellow fever vaccines in a

25   randomized, placebo-controlled trial. Rev Saude Publica
39:413-420; Camacho, L. A., S. Freire Mda, L. Leal Mda, S.
G. Aguiar, J. P. Nascimento, T. Iguchi, A. Lozana Jde, and
R. H. Farias. 2004. Immunogenicity of WHO-17D and Brazilian
17DD yellow fever vaccines: a randomized trial. Rev Saude

Publica-38:671-8).

The attenuated alive virus vaccine of the yellow fever
(FA) 17D strain, constitutes one of the best and safer
vaccines nowadays, having a well established methodology of
5   production and a serious quality control, including the
monkey neurovirulence test. Besides, it promotes lifetime
immunity (Monath, T. 2003. Yellow Fever Vaccine, 4th ed.
W.B. Saunders Company, USA) and it is capable of inducing
both cellular immune and humoral responses (Co, M. D., M.
10   Terajima, J. Cruz, F. A. Ennis, and A. L. Rothman. 2002.
Human cytotoxic T lymphocyte responses to live attenuated
17D yellow fever vaccine: identification of HLA-B35-
restricted CTL epitopes on nonstructural proteins NS1,
NS2b, NS3, and the structural protein E. Virology 293:151-
15   63); in addition to being low cost and one single dose. Its
use was estimated in 400 million doses.

Due to this, its characteristics make it appropriate
for the development of 17D virus as a vaccine expression
vector of the heterolog antigens.
20   But, for the development of the flavivirus, expressing
heterolog antigens, it is necessary to:

(a)   the   sketch   of   strategies   that   allow   the
       introduction   of   heterolog   antigens,   without
       compromise of the structure and replication of the
25   virus;

(b)   ensure that the construction of the cDNA (and the
       RNA transcripts) generate a non-pathogenic virus
       and moreover that the foreign sequence stays
       integrated in the viral genome; and

(c) guarantee that the FA recombinant virus, besides
being   attenuated,   keeps   the   immunologic
properties,   expressing   the   heterolog   antigens,
inserted in a way that it induces the appropriated
immune response. It is also important that the
replication   capacity   in   certified   cells   for
production of vaccines is maintained.

The development of the recombinant DNA technology made
it possible the progress in the studies of structure and
expression of viral RNA genome. To manipulate the genomic
RNA, it is necessary that the complementary DNA become
available. Genetic modifications may be introduced in
determined sites of the viral genome.

The pioneer study of David Baltimore (Racaniello, V.
R., and D. Baltimore. 1981. Cloned poliovirus complementary
DNA is infectious in mammalian cells. Science 214:916-9),
was the first one to demonstrate that it possible to
regenerate virus for the complementary DNA of the
poliomyelitis virus. With the development of efficient
systems *in vitro* transcription, it made it possible to the
complete synthesis of viral RNA viral *in vitro* with
efficiency much greater than the cDNA transcription in the
cell. The development of efficient methods of cells
transfection with nucleic acids, as for example
electroporation and the use of cationic liposome's
contributed to the increase of the transfection efficiency
of cell transfection with RNA and viral regeneration. The
basis of methodology of the infectious clone is established

and has been used to obtain infectious clones to other
virus of the positive strand.

The infectious clones may be used to better understand
the molecular bases of diverse biological phenomena such
as: the virulence, attenuation, mechanism of cell
penetration, replication, relation with the host,
conditional mutant and the design of mutants for the
required functions (Bonaldo, M. C., P. S. Caufour, M. S.
Freire, and R. Galler. 2000. The yellow fever 17D vaccine
virus as a vector for the expression of foreign proteins:
development of new live flavivirus vaccines. Mem Inst
Oswaldo Cruz 95 Suppl 1:215-23; Bonaldo, M. C., R. C.
Garratt, P. S. Caufour, M. S. Freire, M. M. Rodrigues, R.
S. Nussenzweig, and R. Galler. 2002. Surface expression of
an immunodominant malaria protein B cell epitope by yellow
fever virus. J Mol Biol 315:873-85).

The construction of a complete cDNA shape of the 17D
vaccine virus, that can be transcript *in vitro,* producing
RNA infectious virus, was described for the first time by
Rice and colleagues (Rice, C. M., A. Grakoui, R. Galler,
and T. J. Chambers. 1989. Transcription of infectious
yellow fever RNA from full-length cDNA templates produced
by in vitro ligation. New Biol 1:285-96). The virus –
obtained from cDNA – was indistinguished from the parental
virus, the 17D-204 subcepa, by different criteria (Rice, C.
M., A. Grakoui, R. Galler, and T. J. Chambers. 1989.
Transcription of infectious yellow fever RNA from full-
length cDNA templates produced by in vitro ligation. New
Biol 1:285-96).

The acquisition of vaccines shares seeds from cDNA in good production practices was described by the first time by Marchevsky and collaborators (Marchevsky, R. S., J. Mariano, V. S. Ferreira, E. Almeida, M. J. Cerqueira, R.

5   Carvalho, J. W. Pissurno, A. P. da Rosa, M. C. Simoes, and C. N. Santos. 1995. Phenotypic analysis of yellow fever virus derived from complementary DNA. Am J Trop Med Hyg 52:75-80), and later by Galler and Freire (patent documents US 6,171,854 and US 6,859,522) and Freire and collaborators

10   (document of patent BRPI 9804283). The production process described by Freire and collaborators (patent document BRPI 9804283) may also be, in a near future, the modernization of the production of the amarilic vaccine; making it possible a significative increase in the production and

15   improvement of the product quality (Freire, M. S., G. F. Mann, R. S. Marchevsky, A. M. Yamamura, L. F. Almeida, A. V. Jabor, J. M. Malachias, E. S. Coutinho, and R. Galler. 2005. Production of yellow fever 17DD vaccine virus in primary culture of chicken embryo fibroblasts: yields,

20   thermo   and   genetic   stability,   attenuation   and immunogenicity. Vaccine 23:2501-12).

This work created the perspective for the use of the 17D virus as an expression vector for heterolog antigens. There are several ways to obtain an expression vector from

25   the virus with positive string RNA genome, some of which are described in published revisions by our research group (Bonaldo, M. C., P. S. Caufour, M. S. Freire, and R. Galler. 2000. The yellow fever 17D vaccine virus as a vector for the expression of foreign proteins: development

of new live flavivirus vaccines. Mem Inst Oswaldo Cruz 95

Suppl 1:215-23; Galler, R., M. S. Freire, A. V. Jabor, and

G. F. Mann. 1997. The yellow fever 17D vaccine virus:

molecular basis of viral attenuation and its use as an

5   expression vector. Braz J Med Biol Res 30:157-68).

One of the alternatives in which our research group is

working refers to the substitution of the prM/E proteins of

yellow fever by the equivalent proteins of the dengue

virus, so it can be obtained a chimeric virus. This

10   approach has the advantage of the previous immunity against

the vector wouldn't be a limit, since the envelope E

protein contains all the epitops for viral neutralization.

The approach of change of prM/E genes among the

flavivirus was described for the first time in the patent

15   document US6,184,024 and US6,676,936, which described the

new virus with the prM/E genes of dengue 1 or 2 and the

remaining of the virus genome Den 4. The first chimeric

virus from 17D genome was created by change of prM/E genes

of the Japanese encephalitis virus (JE) (Chambers, T. J.,

20   A. Nestorowicz, P. W. Mason, and C. M. Rice. 1999. Yellow

fever/Japanese encephalitis chimeric viruses: construction

and biological properties. J Virol 73:3095-101). This

Chimeric was immunogenic and attenuated in monkeys, so it

could promote a total protection to these animals, in face

25   of a intracerebral challenge (IC) with the wild JE virus

(Monath, T. P., I. Levenbook, K. Soike, Z. X. Zhang, M.

Ratterree, K. Draper, A. D. Barrett, R. Nichols, R.

Weltzin, J. Arroyo, and F. Guirakhoo. 2000. Chimeric yellow

fever virus 17D-Japanese encephalitis virus vaccine: dose-

response effectiveness and extended safety testing in rhesus monkeys. J Virol 74:1742-51). Recently, a clinical study in humans demonstrated that the chimerical vaccine FA/JE is safe and immunogenic in man, in similar levels to

5   the FA 17D, with a high possibility of use, in the future, for the prevention of the Japanese encephalitis in travelers and residents in endemic regions (Monath, T. P. 2002. Japanese encephalitis vaccines: current vaccines and future prospects. Curr Top Microbiol Immunol 267:105-38;

10 Monath, T. P., F. Guirakhoo, R. Nichols, S. Yoksan, R. Schrader, C. Murphy, P. Blum, S. Woodward, K. McCarthy, D. Mathis, C. Johnson, and P. Bedford. 2003. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and

15 immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis 188:1213-30).

Our research group constituted four chimeric virus containing the cDNA of different dengue 2 cepas, and one of

20 these constructions was selected for immunogenicity tests. Theses tests were performed in murine model, the results being published with the characterization of the growth and viral attenuation (Caufour, P. S., M. C. Motta, A. M. Yamamura, S. Vazquez, Ferreira, II, A. V. Jabor, M. C.

25 Bonaldo, M. S. Freire, and R. Galler. 2001. Construction, characterization and immunogenicity of recombinant yellow fever 17D-dengue type 2 viruses. Virus Res 79:1-14).

In this strategy it was also used the creation of a chimeric virus FA 17D for the creation of a tetravalent

vaccine against the different sorotypes of dengue virus
(Guirakhoo, F., J. Arroyo, K. V. Pugachev, C. Miller, Z. X.
Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran,
K. Soike, M. Ratterree, and T. P. Monath. 2001.
Construction, safety, and immunogenicity in nonhuman
primates of a chimeric yellow fever-dengue virus
tetravalent vaccine. J Virol 75:7290-304; Guirakhoo, F., K.
Pugachev, J. Arroyo, C. Miller, Z. X. Zhang, R. Weltzin, K.
Georgakopoulos, J. Catalan, S. Ocran, K. Draper, and T. P.
Monath. 2002. Viremia and immunogenicity in nonhuman
primates of a tetravalent yellow fever-dengue chimeric
vaccine: genetic reconstructions, dose adjustment, and
antibody responses against wild-type dengue virus isolates.
Virology 298:146-59; Guirakhoo, F., K. Pugachev, Z. Zhang,
G. Myers, I. Levenbook, K. Draper, J. Lang, S. Ocran, F.
Mitchell, M. Parsons, N. Brown, S. Brandler, C. Fournier,
B. Barrere, F. Rizvi, A. Travassos, R. Nichols, D. Trent,
and T. Monath. 2004. Safety and efficacy of chimeric yellow
Fever-dengue virus tetravalent vaccine formulations in
nonhuman primates. J Virol 78:4761-75, US Patent Documents
6,696,281 and WO0139802). In tissue culture, these chimera
grow in high degrees, and were immunogenic in inoculated
monks with individual formulations and tetravalent of these
recombinants. But, we may stress that a higher immune
response against one of the recombinant, the chimera
FA/den2, due, probably, to a grater replication rate of
this virus. :

An ideal vaccine against the four sorotypes, as well
as inducing a long-lasting response, should protect the

individual against the four sorotypes efficiently, because an incomplete immunization may unleash the sickness in its more serious form. Later, other formulations were tested in monkeys, with the intention of reducing the dominant 5 immunogenicity of the chimera FA/Den2 (Guirakhoo, F., K. Pugachev, J. Arroyo, C. Miller, Z. X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Draper, and T. P. Monath. 2002. Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric .0 vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 298:146-59). In the meantime, the adjustment of the dose for the chimera den2 resulted, in spite of a more balanced reply against the chimeric viruses types 1, 2 and 15 3, in a more accented reply against the chimera type 4. These results indicate that the development of a tetravalent vaccine should pass by tests with different formulations, so that an ideal adjustment may be obtained to be tested in monkeys before an optimum formulation may 20 be attained to be used in tests of safety and immunogenicity in humans in a phase I clinical study.

The second approach refers to the insertion of the protein epitopes in the virus 17D genome of. Such insertions may be done in very immunogenic proteins of the 25 amarilic virus, through duplication of the processing signals of the viral polyprotein by viral protease and the creation of expression cassettes - as was done with an epitope of ovalbumin, response inductor of the lymphocyte T cytotoxic, that was inserted between the genes NS2B and NS3

(McAllister, A., A. E. Arbetman, S. Mandl, C. Pena-Rossi, and R. Andino. 2000. Recombinant yellow fever viruses are effective therapeutic vaccines for treatment of murine experimental solid tumors and pulmonary metastases. J Virol

5   74:9197-205), Patent Documents US6,589,531 and US20030157128). Immunization of mice with the recombinant virus induced protection against a lethal dose of malignant melanoma cells that expressed the same epitope. It is important that the new viruses be attenuated with the

10 vaccine 17D, that they are genetically stable and retain the immunogenic properties do heterologous antigen, promoting the correct induction of the immune response. In this sense, it should be noted that the expression of the epitope de *Plasmodium yoelii* through its insertion between

15 the NS2B-NS3 genes of the virus 17D (Tao, D., G. Barba-Spaeth, U. Rai, V. Nussenzweig, C. M. Rice, and R. S. Nussenzweig. 2005. Yellow fever 17D as a vaccine vector for microbial CTL epitopes: protection in a rodent malaria model. J Exp Med 201:201-9).

20   It became interesting to test this system for the expression of larger genetic fragments. In this sense, our research group opted to insert the green fluorescent algae genes (GFP). This gene facilitates monitoring the infectiousness of the transcribed RNA *in vitro*, as from

25 plasmidial molds, to allow the direct visualization of the synthesized proteins in transfected cultures through fluorescent microscopy.

        The insertion strategy is described in Figure 3, in which the upper part represents the genomic structure and

the genetic expression. The Flavivirus genome is translated

into a single polyprotein, which is cleaved by cellular

proteases (↓) or viral (▼). Black vertical bars indicate

transmembrane hydrophobic domains, and the asterisks

5   indicate glycosylation sites connected to asparagine.

Shadowed areas in C and prM/E represent as structural

proteins present in the mature infectious viruses. The

lower part presents the general genome structure, the

sequences in the cleavage sites and the proteolytic

10 cleavages necessary for the insertion of the gene reporter

between NS2A and 2B. Such strategy applies to the other

sites cleaved by viral protease, situated between C-prM,

NS2B-3, NS3-4A, NS4A-4B and NS4B-5.

The GFP gene was inserted between NS2A-2B and NS2B-NS3

15 without the recovery of the infectious virus, suggesting

that the insertion of larger genetic fragments in the virus

17D genome through this approach is not possible (Bonaldo

MC and Galler R, data not published).

Another manner of developing recombinant amarylic

20 viruses having various pathogenic epitopes was the

expression of protean epitopes previously classified as

important in some kinds of immune replies, whether humoral

or cellular, by direct insertion in the viral polyprotein.

The different viral proteins contain epitopes related to

25 the induction of the cellular reply (CTL) and humoral

(formation of antibodies), in such a way that there are

different possibilities of optimizing expression and

immunogenicity.

A new version of the FA infectious clone was developed, containing restriction sites in the viral envelope protein gene that allowed the insertion "in-frame" of the heterologous epitopes. This was possible due to the availability of their three-dimensional structure, which allowed an analysis of the areas where insertions would be viable. A site for the insertion of the epitopes was identified in these three-dimensional analyses (f-g loop of the envelope protein), and various epitopes of different microorganisms were already inserted and expressed in the f-g loop, including epitopes de *Plasmodium* sp, dengue and arenavirus (Bonaldo, M. C., R. C. Garratt, M. S. Freire, and R. Galler. 2005. Novel Flavivirus vector useful for expressing heterologous antigens comprises foreign gene sequences inserted at sites in the level of its envelope protein. Great-Britain).

With relation to the *Plasmodium sp* epitopes, a total of 16 new viruses were created, which expressed epitopes related to the response by the T CD4+ or T CD8+ cells or the B cells. A repetitive humoral epitope of the CS surface protein of the sporozoite form of the *P. falciparum was* inserted in the *fg* loop and the virus regenerated. This virus was classified in terms of the culture growth of the cells, neutralization by soros against yellow fever and monoclonal against the epitope, this experiment proved its correct presentation in the viral surface as expected from the three-dimensional modeling, and attenuation and immunogenicity in mice (Bonaldo, M. C., R. C. Garratt, P. S. Caufour, M. S. Freire, M. M. Rodrigues, R. S.

Nussenzweig, and R. Galler. 2002. Surface expression of an immunodominant malaria protein B cell epitope by yellow fever virus. J Mol Biol 315:873-85).

A recombinant Virus 17D expressing an epitope of the

5   *P. yoelii* T CD8 *cell*, through insertion in the f-g loop, also was constructed. This virus did not have its growth *in vitro* characteristics altered, but showed itself more attenuated in the virulence test in mice than the virus vaccine 17DD. This epitope was correctly presented on the

10 viral surface and is immunogenic, based on the results of immunization of mice and the Elispot tests and response with *P.yoelii* sporozoites, response against which was observed a protection of 70%.

Our research group also made a more detailed

15 evaluation of the attenuation of the chimeric viruses, expressing the humoral epitopes *P.falciparum and P.yoelii* T CD8 through the intracerebral inoculation test in rhesus monkeys, in accordance with the requirements established by the World Health Organization for the amarilic virus

20 vaccine. The results suggest that both the viruses are, at the minimum, as attenuated as the 17DD virus vaccine used in human vaccination. A comparative analysis of the virus envelope containing the two insertions showed that the original structural "design" of the insertion, long from

25 the domain III involved in the connection to the receptor/ tropism, was enough to not cause any alteration in the viral virulence, a fundamental aspect in the validation of this approach (Bonaldo, M. C., R. C. Garratt, R. S. Marchevsky, E. S. Coutinho, A. V. Jabor, L. F. Almeida, A.

M. Yamamura, A. S. Duarte, P. J. Oliveira, J. O. Lizeu, L. A. Camacho, M. S. Freire, and R. Galler. 2005. Attenuation of recombinant yellow fever 17D viruses expressing foreign protein epitopes at the surface. J Virol 79:8602-13). This

5   approach constitutes a recently conceded patent (Bonaldo MC, Garrat RC, Freire MS & Galler R (2001) Use of Flaviviruses for the expression of foreign protein epitopes and the development of new live attenuated vaccines for immunization against Flaviviruses and other infectious

10   agents, GB 0105877.5 e PCT PCT/BR02/00036).

A fourth approach in the use of the 17D virus as an expression vector refers to the insertion of genes in the non translated 3´ region (NTR). This approach was done a lot in function of the variability of the length of this

15   region in the FA virus (from Filippis, A. M., R. M. Nogueira, H. G. Schatzmayr, D. S. Tavares, A. V. Jabor, S. C. Diniz, J. C. Oliveira, E. Moreira, M. P. Miagostovich, E. V. Costa, and R. Galler. 2002. Outbreak of jaundice and hemorrhagic fever in the Southeast of Brazil in 2001:

20   detection and molecular characterization of yellow fever virus. J Med Virol 68:620-7; Mutebi, J. P., R. C. Rijnbrand, H. Wang, K. D. Ryman, E. Wang, L. D. Fulop, R. Titball, and A. D. Barrett. 2004. Genetic relationships and evolution of genotypes of yellow fever virus and other

25   members of the yellow fever virus group within the Flavivirus genus based on the 3' noncoding region. J Virol 78:9652-65).

This methodology was described by Andino and collaborators (Andino, P.R., Mcallister, M.N., 2002,

Recombinant Bicistronic Flaviviruses and Methods of Use Thereof, WO 02/089840) and, basically, involved the creation of restriction sites for the insertion of expression modules. These modules, for their part, were

5 constituted of a sequence derived from the enterovirus (Mengo or poliovirus) or from a Pest virus (Bovina Diarrhea virus), to which is directed the connection of the ribosomal sub-units in a manner that the translation of the heterologous gene may happen almost at the 3´ NTR

10 extremity, without needing a start in the 5´ NTR region, as is characteristic of eukaryotic RNA. In this manner, the viral RNA acts as a bi-cystronic messenger, allowing the initiation of protein synthesis as from 2 RNA points, independently of the viral protein synthesis. These

15 sequences are known as internal ribosome entry sites (IRES) Such modules vary in size, depending on the origin of the IRES and the heterologous gene to be expressed.

Figure 4 represents the insertion of the heterologous sequences in the 3´ NTR regions of the 17D virus. The

20 insertions of the Mengo enterovirus IRES (569 nt) and polio (663 nt) were done through cloning in restriction sites (AscI and NotI), which are adjacent to the protein P24 (693 nt) gene sequence of the human 1 immunodeficiency virus (through the NotI and PacI enzymes). The total length of

25 the insertions varied from 1090 to 1356 nt. The restriction sites were initially introduced, as a set (AscI, NotI and PacI), exactly 25 nucleotides after the termination codon (nucleotide 10379 as from the 5´ extremity).

The transfection of the Vero in culture cells with RNA

transcription *in vitro*, as from the cADN molds, allowed the viral regeneration referent to the constructions traced out in Figure 3. Analysis of the resulting virus genomes, by means of nucleotide sequencing of the amplification products of this region, showed the elimination of the nucleotides. In the case of the construction with the Mengo virus IRES, the genetic instability became evident early in the first pass. The 17D-IRES-P24 virus present floating on the culture surface, presenting a cytopathic effect, had lost part of the 3´ NTR region. The termination codon remained like that as well as the first 25 nucleotides that extended up to the AscI site and more than the 22 initial IRES nucleotides. 1437 nucleotides were eliminated from this point, leaving only the last 339 nucleotides (from 508) in this region of the 17D virus. In the case of the 17D-IRES-Polio-P24 virus, the genetic instability was demonstrated by the sequencing of the 3´ NTR region of the virus present on the surface of the second pass in Vero cells. The termination codon remained intact in the genome of this virus and the first 19 nucleotides after it, following the elimination of a total of 1398 nucleotides, including the IRES and P24. The last 484 nucleotides of the original 17D virus 3´NTR region remained intact. This data showed that instability of the longer insertions in this genome region.

The genetic instability of insertions in the Flavivirus genome in the 3´ NTR region is also corroborated by the data of Pierson and collaborators (Pierson, T. C., M. S. Diamond, A. A. Ahmed, L. E. Valentine, C. W. Davis,

M. A. Samuel, S. L. Hanna, B. A. Puffer, and R. W. Doms.
2005. An infectious West Nile virus that expresses a GFP
reporter gene. Virology 334:28-40), to obtain the insertion
of the expression modules similar to that described above,
5   but using the GFP gene as an indicator of viral
replication. Various virals isolated, analyzed after 2
passes in culture cells, led to the loss of the nucleotides
that compose the IRES, as well as part do gene that codes
the GFP.

10   The sixth possible approach in the use of the FA 17D
virus for the expression of heterologous antigens refers to
the development of replicons. These molecules correspond to
parts of the viral genome from which the structural genes
necessary for the production of viral particles were
15   removed, although they maintained all the elements
necessary for the replication of the RNA in itself. The
amplification of the RNA in the transfected cells cytoplasm
allows the transitory expression of heterologous genes,
expression that suggests the possibility of the in
20   vaccination (Harvey, T. J., W. J. Liu, X. J. Wang, R.
Linedale, M. Jacobs, A. Davidson, T. T. Le, I. Anraku, A.
Suhrbier, P. Y. Shi, and A. A. Khromykh. 2004.
Tetracycline-inducible packaging cell line for production
of Flavivirus replicon particles. J Virol 78:531-8;
25   Khromykh, A. A. 2000. Replicon-based vectors of positive
strand RNA viruses. Curr Opin Mol Ther 2:555-69; Tannis, L.
L., A. Gauthier, C. Evelegh, R. Parsons, D. Nyholt, A.
Khromykh, and J. L. Bramson. 2005. Semliki forest virus and
Kunjin virus RNA replicons elicit comparable cellular

immunity but distinct humoral immunity. Vaccine 23:4189-94; Westaway, E. G., J. M. Mackenzie, and A. A. Khromykh. 2003. Kunjin RNA replication and applications of Kunjin replicons. Adv Virus Res 59:99-140). Jones and

5   collaborators (Jones, C. T., C. G. Patkar, and R. J. Kuhn. 2005. Construction and applications of yellow fever virus replicons. Virology 331:247-59) recently described a series of replicons based on the 17D virus genome. These replicons consist of the 17D virus genome deprived of the structural

10 region that codifies the genes of the C-prM-E proteins (nucleotides 179 to 2382). Only the first 21 amino acids of C and the last 24 residues of E were kept. Three heterologous genes were inserted and expressed in the replicons in a manner dependent on the RNA replication,

15 substituting the structural gene sequences. Meanwhile, no evidence of genetic stability of the heterologous genes, as well as studies on the immunogenicity of their products has been approached. The expression levels of the heterologous proteins also were not specified, in a way that use of this

20 system for the development of new vaccines was not established. The principal applications of this expressions system, based on the 17D virus genome, are limited to studies on RNA viral replication mechanisms, RNA packaging and formation of viral particles.

25   It should be considered that the various methodologies described in this document for the insertion and expression of heterologous genes into recombinants flavivirus, as well as the object of this document, are also approaches with broad application in the expression of

the whole or part of the viral genome in plasmids and DNA
and RNA replicons, or even in other non-infective or
infective viral systems. Khromykh, A.A., Westaway, E.G.,
1997. Subgenomic replicons of the flavivirus Kunjin:
construction and applications. J. Virol. 71 (2), 1497-
1505; Kofler, R.M., Aberle, J.H., Aberle, S.W., Allison,
S.L., Heinz, F.X., Mandl, C.W., 2004. Mimicking live
flavivirus immunization with a noninfectious RNA vaccine.
Proc. Natl. Acad. Sci. U.S.A. 101, 1951-1956; Aberle, J.H.,
Aberle, S.W., Kofler, R.M., Mandl, C.W., 2005. Humoral and
cellular immune response to RNA immunization with
flavivirus replicons derived from tick-borne encephalitis
virus. J. Virol. 79, 15107-15113; Aleshin, S.E., Timofeev,
A.V., Khoretonenko, M.V., Zakharova, L.G., Pashvykina,
G.V., Stephenson, J.R., Shneider, A.M., Altstein, A.D.
2005. Combined prime-boost vaccination against tick-borne
encephalitis (TBE) using a recombinant vaccinia virus and a
bacterial plasmid both expressing TBE virus non-structural
NS1 protein. BMC Microbiology 5:45-49; Konishi, E., Kosugi,
S., Imoto, J. 2006. Dengue tetravalent DNA vaccine inducing
neutralizing antibody and an amnestic responses to four
serotypes in mice Vaccine 24: 2200-2207; Mason, P.W.,
Shustov, A.V., Frolov, I. 2006). Production and
characterization of vaccines based on flaviviruses
defective in replication. Virology 351 432-443.

The seventh and last possible approach up to the
moment, using the FA 17D virus as an expression vector,
refers to the object of this current invention. In this
case, given the impossibility of regenerating 17D viruses

containing insertions longer than viral epitopes (> 36 amino acids), whether in inter-genetic regions cleaved by viral protease or in the 3´NTR region, our group established a new approach for this purpose. This

5   alternative is based on the insertion of the heterologous sequences - including, but not limited to those of the 10 to 2000 nucleotides - between the genes that code the E and NS1 proteins of the 17D virus. This approach is similar, theoretically, to the insertion between genes that code

10 proteins cleaved by viral protease. Meanwhile, the cleavage between E and NS1 is done by a cellular enzyme (signalase) present in the endoplasmatic reticule, in such a manner that the cleavage sites and other structural elements necessary of viral viability are different, constituting a

15 novelty in this methodology.

The endoplasmatic reticule serves as an entrance port for the proteins destined to all the compartments of the secreting via, that is, for the plasmatic membrane, the cell exterior and endocytic organelles. The majority of the

20 membrane proteins and secreting via are co-traductionally integrated in the RE membrane, or pass by this to the RE lumen via specific membrane sites.

The addressing of the proteins to the RE is triggered by the presence of signal sequences in these proteins. The

25 signal sequences are highly degenerated and essentially, uncharged, with a predominance of hydrophobic residues, and with an average size of 7 to 12 protein amino acids (von Heijne, G. 1990. The signal peptide. J Membr Biol 115:195-201).

In a first stage, the signal sequence is recognized, beginning to emerge from the tunnel exit of the ribosome during the proteic translation, by a signal recognition particle, of a ribonucleoproteic nature (SRP: "signal recognition particle); (Halic, M., and R. Beckmann. 2005. The signal recognition particle and its interactions during protein targeting. Curr Opin Struct Biol 15:116-25; Walter, P., and A. E. Johnson. 1994. Signal sequence recognition and protein targeting to the endoplasmic reticulum membrane. Annu Rev Cell Biol 10:87-119). Then a connection of the motif to a hydrophobic split occurs composed of a group of methionines in the SRP 54 kDa sub-unit (Keenan, R. J., D. M. Freymann, P. Walter, and R. M. Stroud. 1998. Crystal structure of the signal sequence binding subunit of the signal recognition particle. Cell 94:181-91; Lutcke, H., S. High, K. Romisch, A. J. Ashford, and B. Dobberstein. 1992. The methionine-rich domain of the 54 kDa subunit of signal recognition particle is sufficient for the interaction with signal sequences. Embo J 11:1543-51; Zopf, D., H. D. Bernstein, A. E. Johnson, and P. Walter. 1990. The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be cross linked to a signal sequence. Embo J 9:4511-7). In eukaryotes, this association causes a delay in the elongation of polypeptide synthesis during the translation process. This complex connects itself to the RE membrane by a specific receptor (Keenan, R. J., D. M. Freymann, R. M. Stroud, and P. Walter. 2001. The signal recognition particle. Annu Rev Biochem 70:755-75). Both the

SRP complex receptor - signal peptide and the SRP are GTPases (Egea, P. F., S. O. Shan, J. Napetschnig, D. F. Savage, P. Walter, and R. M. Stroud. 2004. Substrate twinning activates the signal recognition particle and its

5   receptor. Nature 427:215-21; Focia, P. J., I. V. Shepotinovskaya, J. A. Seidler, and D. M. Freymann. 2004. Heterodimeric GTPase core of the SRP targeting complex. Science 303:373-7), that undergo reciprocal activation, causing the signal peptide to be released from the

10   addressing complex and taken to the ribosome tunnel exit alignment, as to the aquatic entrance channel of the RE protein, or translocon (Beckmann, R., C. M. Spahn, N. Eswar, J. Helmers, P. A. Penczek, A. Sali, J. Frank, and G. Blobel. 2001. Architecture of the protein-conducting

15   channel associated with the translating 80S ribosome. Cell 107:361-72; Menetret, J. F., A. Neuhof, D. G. Morgan, K. Plath, M. Radermacher, T. A. Rapoport, and C. W. Akey. 2000. The structure of ribosome-channel complexes engaged in protein translocation. Mol Cell 6:1219-32).

20   The translocons are comprised of various RE membrane proteins that associate themselves in such a manner as to form an aqueous pore, through which secreted proteins and domain protein lumen from the membrane pass from the cytosol to the RE (Johnson, A. E., and M. A. van Waes.

25   1999. The translocon: a dynamic gateway at the ER membrane. Annu Rev Cell Dev Biol 15:799-842). The translocon has an important role in the integration of the membrane proteins (Do, H., D. Falcone, J. Lin, D. W. Andrews, and A. E. Johnson. 1996. The cotranslational integration of membrane

proteins into the phospholipid bi-layer is a multi-step process. Cell 85:369-78; Heinrich, S. U., W. Mothes, J. Brunner, and T. A. Rapoport. 2000. The Sec61p complex mediates the integration of a membrane protein by allowing

5 lipid partitioning of the transmembrane domain. Cell 102:233-44; Higy, M., T. Junne, and M. Spiess. 2004. Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry 43:12716-22; Martoglio, B., and B. Dobberstein. 1995. Protein insertion into the membrane of

10 the endoplasmic reticulum: the architecture of the translocation site. Cold Spring Harb Symp Quant Biol 60:41-5; Mothes, W., S. U. Heinrich, R. Graf, I. Nilsson, G. von Heijne, J. Brunner, and T. A. Rapoport. 1997. Molecular mechanism of membrane protein integration into the

15 endoplasmic reticulum. Cell 89:523-33), therefore, in the topology of these proteins. The mechanism by which the topology of a protein is directed by the cellular translocation machinery is complex. Thus, a protein with a single membrane domain needs to translocate certain RE

20 Lumen domains, leave others in the cytosol and guide the transmembrane segment and move the aqueous utranslocation channel to the lipidic bi-layer. Characteristics such as size and hydrophobic of the transmembrane segments, Charge distribution of the regulatory residues and size and state

25 of the binding regulatory residues may affect the protein topology in the membrane (Beltzer, J. P., K. Fiedler, C. Fuhrer, I. Geffen, C. Handschin, H. P. Wessels, and M. Spiess. 1991. Charged residues are major determinants of the transmembrane orientation of a signal-anchor sequence.

J Biol Chem 266:973-8; Gafvelin, G., M. Sakaguchi, H. Andersson, and G. von Heijne. 1997. Topological rules for membrane protein assembly in eukaryotic cells. J Biol Chem 272:6119-27; Higy, M., T. Junne, and M. Spiess. 2004.

5   Topogenesis of membrane proteins at the endoplasmic reticulum. Biochemistry 43:12716-22; Parks, G. D., and R. A. Lamb. 1991. Topology of eukaryotic type II membrane proteins: importance of N-terminal positively charged residues flanking the hydrophobic domain. Cell 64:777-87;

10   Sakaguchi, M., R. Tomiyoshi, T. Kuroiwa, K. Mihara, and T. Omura. 1992. Functions of signal and signal-anchor sequences are determined by the balance between the hydrophobic segment and the N-terminal charge. Proc Natl Acad Sci U S A 89:16-9; Spiess, M. 1995. Heads or tails--

15   what determines the orientation of proteins in the membrane. FEBS Lett 369:76-9; von Heijne, G. 1989. Control of topology and mode of assembly of a polytopic membrane protein by positively charged residues. Nature 341:456-8; Wahlberg, J. M., and M. Spiess. 1997. Multiple determinants

20   direct the orientation of signal-anchor proteins: the topogenic role of the hydrophobic signal domain. J Cell Biol 137:555-62).

At the translocon entrance, the signal peptide is guided in relation to the membrane to the start of the

25   translocation of its N- or C-terminal sequence through the membrane. The hydrophilic fraction of the polypeptide is transferred then, by the aqueous channel to the RE lumen, and the signal released laterally in the lipidic membrane. On the other side, other protein segments may stop or

restart their transference to the RE or integrate
themselves to the RE lipidic bi-layer as transmembrane
domains (TM), and may generate proteins with multiple
insertions of alpha helices in the lipidic bi-layer (Higy,

5   M., T. Junne, and M. Spiess. 2004. Topogenesis of membrane
proteins at the endoplasmic reticulum. Biochemistry
43:12716-22). The TM domains that promote integration to
the membrane generally consist of 20 to 25 non polar amino
acids, a size sufficient to transpass the membrane lipidic

10   bi-layer.

Figure 5 is referent to the processing of the
Flavivirus polyprotein by cellular and viral proteases. In
(A), viral polyprotein protelic sites for generation of the
structural proteins, and non structural viral envelope

15   components involved in the viral replication process. The
stars (*) represent the glycosilation connected to the
asparagine of certain vital proteins, the grey arrows
highlight the signal peptidase cleavage sites, and the gray
triangles represent the sites for the proteolysis of the

20   viral proteolytic complex (NS2B/NS3). The (?) symbol
represents the cleavage point between the NS1/NS2A viral
proteins, in which acts a still undetermined cellular
protease. The prM protein is later processed by the furine
protease in the release of the cell viral particle

25   (Stadler, K., Allison, S.L., Schalich, J. and Heinz, F.X.
1997. Proteolytic activation of tick-borne encephalitis
virus by furin. J Virol. 71:8475-8481). In (B), topology of
the prM and E structural protein membranes, which are
translocated to the cellular RE and are found associated to

their membrane by means of two domains of transmembranar
helices, that are indicated by cylinders. The signalase
cleavage sites and the NS2B/NS3 viral protease are signed
according to the nomenclature below the figure.

5   In Flavivirus, the polyprotein viral precursor of the
structural and non structural proteins pass through the RE
membrane at various points and are processed thus: on the
lumen side of the RE membrane, by the cellular enzymes,
signalases, and on the cytoplasmic side, by the NS2B/NS3

10   proteolytic viral complex, (Figure 5A). The RE and the
viral particle assembly site, which are formed by the
transport of the virions to the cell exterior, by means of
the exotic or secretory via (Mackenzie, J. M., and E. G.
Westaway. 2001. Assembly and maturation of the flavivirus

15   Kunjin virus appear to occur in the rough endoplasmic
reticulum and along the secretory pathway, respectively. J
Virol 75:10787-99).

       Cleavage of the polyprotein in the C/prM, prM/E and
E/NS1intergenic sites, done by signalase, generate the prM

20   and E structural proteins, that remain anchored in the
luminal face of the RE membrane and form the flavivirus
viral envelope. The prM and E proteins of the flavivirus
envelope are type I membrane proteins (Higy, M., T. Junne,
and M. Spiess. 2004. Topogenesis of membrane proteins at

25   the endoplasmic reticulum. Biochemistry 43:12716-22;
Paetzel, M., A. Karla, N. C. Strynadka, and R. E. Dalbey.
2002. Signal peptidases. Chem Rev 102:4549-80); That is,
the translocation of these proteins to the RE lumen is
started by the amino extremity of the polypeptide chain,

which associates itself to the translocon, undergoing
cleavage by signalase. This leads to the removal of the
signal peptide and consequent release of the processed N-
terminal from the protein to the RE lumen RE (Figure 5 B).

5   The prM and E proteins are anchored by their carboxi-
terminal in the cellular and viral membranes. These domains
are composed of two hydrophobic stretches separated by a
small fragment containing ate least one hydrophobic
residue. Thus, on the side of the RE lumen, prM and E form

10   a stable heterodimer that will form the viral envelope
(Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F.
X. Heinz. 1995. Synthesis and secretion of recombinant
tick-borne encephalitis virus protein E in soluble and
particulate form. J Virol 69:5816-20; Konishi, E., and P.

15   W. Mason. 1993. Proper maturation of the Japanese
encephalitis virus envelope glycoprotein requires
cosynthesis with the premembrane protein. J Virol 67:1672-
5; Lorenz, I. C., S. L. Allison, F. X. Heinz, and A.
Helenius. 2002. Folding and dimerization of tick-borne

20   encephalitis virus envelope proteins prM and E in the
endoplasmic reticulum. J Virol 76:5480-91). Thus, the prM
and E viral envelope proteins have two transmembrane
domains (TM1 and 2; figure 5, panel B), which promote their
association to the lipidic bi-layer, the first, in the

25   direction amino to the carboxi terminal of the polypeptide
chain, consists of a sequence of transference stops of the
protein to the RE lumen, and the second, from the signal
sequence for importation and processing in the RE.

The two TM domains of the E and prM proteins form anti-parallel alpha-helices, without contact between themselves, which cross the RE Lumen membrane to the cytoplasm and Lumen again (Figure 5, panel B). For their

5   part, the fragment of 4 to 6 amino acids, rich in polar residues that serve as a connection between these two TM domains, appear to be associated to the internal layer of the phospholipid polar groups of the membrane (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz.

10 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J Virol 73:5605-12; Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3:13-22; Stiasny, K., S. L.

15 Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142-7; Zhang, W., P. R. Chipman, J. Corver, P. R. Johnson, Y. Zhang, S. Mukhopadhyay, T. S.

20 Baker, J. H. Strauss, M. G. Rossmann, and R. J. Kuhn. 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat Struct Biol 10:907-12).

The protein of capsid (C) is separated from the prM, precursor protein of the membrane protein or M, by a signal

25 sequence that directs the translation of the prM. Meanwhile, so that cleavage of the peptide signal occurs and formation of the COOH terminal of the C protein C and the prM N-terminal, it is strictly necessary that the NS2B/NS3 proteolytic complex first catalyzes the COOH

terminal COOH of the C protein on the cytoplasmatic side of
the RE membrane RE (Figure 5 B). This is the only site of
the polyprotein region containing the structural proteins
that are processed by this enzyme (Amberg, S. M., A.

5   Nestorowicz, D. W. McCourt, and C. M. Rice. 1994. NS2B-3
proteinase-mediated processing in the yellow fever virus
structural region: in vitro and in vivo studies. J Virol
68:3794-802; Lobigs, M. 1993. Flavivirus premembrane
protein cleavage and spike heterodimer secretion require

10 the function of the viral proteinase NS3. Proc Natl Acad
Sci U S A 90:6218-22; Yamshchikov, V. F., and R. W.
Compans. 1993. Regulation of the late events in flavivirus
protein processing and maturation. Virology 192:38-51). It
is only after this cleavage that the cleavage of the signal

15 peptide by the signal peptidase happens, probably due to
the conversion of the cleavage signal peptidase site from a
cryptic conformation to an accessible one (Lobigs, M. 1993.
·Flavivirus premembrane protein cleavage and spike
heterodimer secretion require the function of the viral

20 proteinase NS3. Proc Natl Acad Sci U S A 90:6218-22). The
cleavage process of the prM protein signal peptide by the
signal peptidase is modulated by the initial hydrolysis of
the C protein C-terminal by viral protease. Thus, it is
only after the cleavage and generation of the mature C

25 protein that the hydrolysis of the signal peptide occurs,
and the consequent release of the prM protein N-terminal in
the RE lumen. This stage is preserved between the
Flavivirus, indicating its regulatory nature during the
processing of the polyprotein structural region (Amberg, S.

M., and C. M. Rice. 1999. Mutagenesis of the NS2B-NS3-
mediated cleavage site in the Flavivirus capsid protein
demonstrates a requirement for coordinated processing. J
Virol 73:8083-94; Stocks, C. E., and M. Lobigs. 1998.

5   Signal peptidase cleavage at the flavivirus C-prM junction:
dependence on the viral NS2B-3 protease for efficient
processing requires determinants in C, the signal peptide,
and prM. J Virol 72:2141-9). In this sense, it was shown
that this coordinated processing is critical for the

10   incorporation of the nucleocapsid during the formation of
the viral particles in the RE (Lee, E., C. E. Stocks, S. M.
Amberg, C. M. Rice, and M. Lobigs. 2000. Mutagenesis of the
signal sequence of yellow fever virus prM protein:
enhancement of signalase cleavage In vitro is lethal for

15   virus production. J Virol 74:24-32; Lobigs, M., and E. Lee.
2004. Inefficient signalase cleavage promotes efficient
nucleocapsid incorporation into budding flavivirus
membranes. J Virol 78:178-86; Stocks, C. E., and M. Lobigs.
1998. Signal peptidase cleavage at the flavivirus C-prM

20   junction: dependence on the viral NS2B-3 protease for
efficient processing requires determinants in C, the signal
peptide, and prM. J Virol 72:2141-9). Therefore, for
coordination of the cytosolic cleavages, and the RE lumen
RE in the C/prM junction, it is indispensable that an

25   efficient incorporation of the nucleocapsid to the
membranes containing the viral envelope proteins occurs,
because the brewing of the subviral particles, containing
only the viral envelope proteins, do not depend on the C
protein or the assembly of the nucleocapsid (Allison, S.

L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.

1995. Synthesis and secretion of recombinant tick-borne

encephalitis virus protein E in soluble and particulate

form. J Virol 69:5816-20; Lorenz, I. C., S. L. Allison, F.

5   X. Heinz, and A. Helenius. 2002. Folding and dimerization

of tick-borne encephalitis virus envelope proteins prM and

E in the endoplasmic reticulum. J Virol 76:5480-91).

The C-terminal portion of the prM protein contains two

adjacent hydrophobic stretches, interrupted by a charged

10 residue; that act, the first transmembrane stretch, as a

stop signal for the prM transference, and the second , as

a signal sequence for the translocation of the E protein to

the RE   (Markoff, L. 1989. In vitro processing of dengue

virus structural proteins: cleavage of the pre-membrane

15 protein. J Virol 63:3345-52; Ruiz-Linares, A., A. Cahour,

P. Despres, M. Girard, and M. Bouloy. 1989. Processing of

yellow fever virus polyprotein: role of cellular proteases

in maturation of the structural proteins. J Virol 63:4199-

209). Two adjacent transmembrane sequences act in the same

20 manner, through the stoppage of the E protein translocation

and the entrance of the RE from the NS1 protein. In a

general fashion, the processing by signal peptidases is

important for the importation of the prM, E and NS1

proteins to the RE, and for the generation of their extreme

25 N-terminal.

Cocquerel   and   collaborators   (Cocquerel,   L.,   C.

Wychowski, F. Minner, F. Penin, and J. Dubuisson. 2000.

Charged residues in the transmembrane domains of hepatitis

C virus glycoproteins play a major role in the processing,

sub-cellular localization, and assembly of these envelope proteins. J Virol 74:3623-33), when they analyzed the C-terminal sequences of the Flavivirus viral envelope proteins, could demonstrate that this organization is very similar to that found in the Hepatitis C virus and in other members of the Flaviviridae Family. It can also be determined, that the sequences which connect the two TM domains, within the different groups, have specific standards related to these different virus groups; but the presence of at least one positively charged group (R or K) in this region was general, indicating an important function. The comparison of this fragment between different virus groups of the Flaviviridae family point to a wide variability of the amino acid sequences of the connection segment of the TM domains TM between these different groups, indicating that these should be related to molecular interactions that would occur specifically within these groups (Cocquerel, L., C. Wychowski, F. Minner, F. Penin, and J. Dubuisson. 2000. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, sub-cellular localization, and assembly of these envelope proteins. J Virol 74:3623-33). Notably, the connection segments of the TM segments of the structural proteins in Flavivirus are longer than their counterparts in other groups, presenting various polar residues preserved (N, Q, S and/or T). Another characteristic consists of the fact that the second Flavivirus TM domain is noticeably larger, with around 19 residues, in relation to the other viral groups of the

family, with around 12 to 13 residues. Mutations in the prM and E TM domains affect the formation of the subviral particles or effective viruses, but appear not to affect the heterodimerization capacity of the prM and E proteins,

5 indicating that these domains are sensitive to a change in their amino acid sequence, and the interactions between the alpha helices of the domains have a role in the formation of the viral envelope (Op De Beeck, A., R. Molenkamp, M. Caron, A. Ben Younes, P. Bredenbeek, and J. Dubuisson.

10 2003. Role of the transmembrane domains of prM and E proteins in the formation of yellow fever virus envelope. J Virol 77:813-20). Recently, it could be established that the chimeric proteins, expressing these Flavivirus prM and E protein transmembrane domains, situated themselves mainly

15 in the RE, indicating that these domains contain retention signals in the RE. It is probable that accumulation of these proteins in the RE occurs, leading to the heterodimerization of these and the brewing of the immature viral particles in the RE lumen, as from which will start

20 the secretion via of the virions to the extra-cellular medium.

In relation to the Flavivirus E protein, these TM domains make part of other structural elements situated in the last one hundred amino acid residues of the C-terminal

25 of this protein, a region denominated stem-anchor (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J Virol 73:5605-12). This region is not part of

the three-dimensional structure elucidated for the E protein ectodomain of different Flaviviruses, due to its hydrophobic character (Modis, Y., S. Ogata, D. Clements, and S. C. Harrison. 2003. A ligand-binding pocket in the

5 dengue virus envelope glycoprotein. Proc Natl Acad Sci U S A 100:6986-91; Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison. 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375:291-8). In the TBE virus E protein, the stem-anchor

10 region covers the residues from 401 to 496 (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J Virol 73:5605-12; Stiasny, K., S. L. Allison, A. Marchler-Bauer,

15 C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142-7)

The stem region connects the E protein ectodomain with the transmembrane region. This domain is composed of two

20 alpha-helices, denominated H1 and H2, separated by a connection sequence (CS) highly preserved in the Flavivirus, see Figure 7A (Stiasny, K., Allison, S.L., Marchler-Bauer, A., Kunz, C. and F.X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements

25 in the envelope glycoprotein of tick-borne encephalitis virus. J. Virol. 70: 8142-8147; Allison, S.L., Stiasny, K., Stadler, K., Mandl, C.W. and F.X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J. Virol. 73, 5605-

5612). The first helix, H1, forms an angle with the external layer of membrane lipids and the second, H2 finds itself placed above the side of the external membrane, with the hydrophobic side turned to the hydrophobic side of the membrane (Mukhopadhyay, S., R. J. Kuhn, and M. G. Rossmann. 2005. A structural perspective of the flavivirus life cycle. Nat Rev Microbiol 3:13-22; Zhang, W., P. R. Chipman, J. Corver, P. R. Johnson, Y. Zhang, S. Mukhopadhyay, T. S. Baker, J. H. Strauss, M. G. Rossmann, and R. J. Kuhn. 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat Struct Biol 10:907-12). It is postulated that the stem region makes contact with the side of the E protein closest to the lipidic membrane, neutralizing the electrostatic repulsion between the phospholipid radicals of the external lipidic membrane and the interior surface of the E protein ectodomain (Zhang, Y., W. Zhang, S. Ogata, D. Clements, J. H. Strauss, T. S. Baker, R. J. Kuhn, and M. G. Rossmann. 2004. Conformational changes of the flavivirus E glycoprotein. Structure (Camb) 12:1607-18). The H1 region appears to be involved in the formation of E protein homotrimers during the fusion process (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J Virol 73:5605-12). In this way, truncated proteins lacking the stem-anchor domains are secreted as dimers, undergo dissociation in acid pH, which causes the fusion process, but does not manage to form trimers. On the other side, proteins

truncated immediately after H1 may form trimers in low pH, indicating that this region may be involved in the conversion of monomers to trimers during the fusion process to the endosomic membrane. The second stem element, CS, is highly preserved in Flavivirus (Stiasny, K., S. L. Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142-7), indicating a still undefined important function.

The second anphipatic element of the stem – H2, jointly with the first transmembrane domain (TM1), are important for the stability of the prM/ E dimer and may be interacting directly with prM.

As was previously discussed, the two TM1 and TM2 transmembrane elements of the E protein C-terminal constitute a membrane double anchor. The TM2 domain appears to be dispensable in the formation of subviral particles (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. J Virol 73:5605-12), meanwhile it is an important functional component in the formation of viral particles and viral infection, because it functions as a signal peptide for the translocation of the NS1 protein to the RE lumen.

SUMMARY OF THE INVENTION

The object of the current invention is the development of a vaccine virus, in especial a Flavivirus vaccine,

obtained from a cloned viral cDNA, having phenotypical
characteristics of attenuation and immunogenicity, and that
is capable of expressing and inducing a response immune to
proteins or fragments of heterologous proteins.

5   The first discovery of the current invention is
related to a method for the production of the recombinant
virus containing sequences of codifying nucleotides of all
or part of the heterologous proteins, characterized by the
following steps:

10   a) modification of the heterologous sequences in such
             a manner that they when cloned and expressed in
             the vector virus, they have in their 5´ portion,
             nucleotides present at the extreme 5´ of the NS1
             gene of this vector virus or the other viruses or
15   functionally equivalent sequences, and in their 3´
             portion, the genomic region corresponding to all
             or part of the stem and anchor domains of the E of
             this vector virus or other viruses functionally
             equivalent sequences, and thus do not compromise
20   the structure and the replication of said vector
             virus;

       b) insertion of the modified heterologous sequences
             in (a) in the intergenic region at the E protein
             structural level and of the non structural NS1 of
25   the vector virus;

       c) obtaining the non pathogenic recombinant virus and
             holder of the immunological properties, containing
             the heterologous sequences stably integrated in
             the viral genome according to the insertion in the

region described in (b) and, like this, expressing the heterologous antigen in such a way that it induces the appropriate immune response.

The second discovery of the current invention is referent to a DNA construction, which consists essentially of (i) a vector itself; (ii) a genetically stable virus genome, in which will be inserted modified heterologous sequences; and (iii) the said modified heterologous sequences and introduced into an insertion site in the intergenic region at the E protein structural and the NS1 non structural viral level during stage (a) of the method cited above.

The third discovery of this invention is associated to the recombinant virus produced according to the above cited method, which contains sequences of codifying nucleotides of all or part of the modified heterologous proteins according to stage (a) of the current invention's method and inserted in the intergenic region at the E protein structural and the NS1 non structural of the vector virus stably integrated into the viral genome; for not being pathogenic; for having immunological properties and for expressing the heterologous antigen in a manner that it induces an appropriate immune response, directed to the vector virus or virulent forms homologous to it and the exogenous protein expressed by it.

The fourth discovery of the current invention corresponds to the vaccine composition to immunize against the vector virus of virulent forms homologous to it and/or other pathogens, of which the gene of the heterologous

protein, expressed by the recombinant virus originated, to which it is constituted, principally, by the said virus obtained according to the above cited method.

BRIEF DESCRIPTION OF DRAWINGS

5   **Figure 1**: Genome organization of Flaviviruses.

Figure 2: Scheme of structural organization of Flaviviruses, representing the viral particle under its immature intracell and mature extracell forms.

Figure 3: Strategy for inserting a reporter gene into FA
10   17D virus genome in the intergenic regions processed by NS2B/NS3 viral protease.

Figure 4: Insertion of heterologous sequences in the 3´ NTR region of 17D virus.

Figure 5: Processing of polyprotein of flaviviruses by cell
15   and virus proteases.

Figure 6: Cleavage point of the signal peptidase in the E and NS1 intergenic region of the flaviviruses.

Figure 7: Comparison of E and EGFP protein topology cloned and expressed in the intergenic region between E and NS1
20   proteins, in the membrane of ER in a recombining flavivirus.

Figure 8: Regions of E and NS1 protein used in the assembly of the cassette of EGFP protein Expression, at FA 17D infectious clone.

25   **Figure 9**: Sequence of amino acids foreseen for heterologous insertion, containing the gene of EGFP cloned in the E/NS1 intergenic region.

Figure 10: Map of the T3 Esa EGFP recombining plasmid.

Figure 11: Analysis of the Vero cells monolayer infection kinetics by the 17D /Esa/5.1$_{glic}$ virus by confocal microscopy.

Figure 12: Comparative diagram of the genome region, comprised between prM and NS1 proteins, in virus of 17D vaccinal phenotype and recombining 17D/Esa/5.1glic, and the respective genome positions.

Figure 13: Propagation properties of the recombining 17D/Esa/5.1$_{glic}$ FA virus in comparison to vaccinal 17D/14 and 17DD Vero cells monolayers .

Figure 14: Analysis of the EGFP fluorescent protein expression kinetics by the 17D/Esa/5.1$_{glic}$ recombining virus in Vero cells and by flow cytometry.

Figure 15: Degree of protection afforded by immunization of BABL/c mice with the 17D /Esa/5.1$_{glic}$T3 virus, on the challenge through intracerebral inoculation with 6.000 PFU of the virus of yellow fever vaccinal strain 17DD.

Figure 16: 0.8% agarose gel electrophoreses analysis of obtained fragments by PCR reactions of T3 and T3 Esa EGFP plasmids and viral RNA preparations of control 17D/E200 and recombinant 17D/Esa/5.1$_{glic}$ viruses. Schematic illustrations of potential experimental synthesis resulting from direct replications of 288 nucleotides that occur in the genome of recombinant 17D/Esa/5.1glic virus.

Figure 17: Genetic stability of 17D/Esa/5.1 glic virus after ten serial passages in Vero cell monolayers. Analysis of two independent series of serial passages, using RT-PCR and FACS methods.

Figura 18: Genetic stability of viral 6 clone, purified by

lyse plaque isolation of 17D/Esa/5.1 glic virus, and submitted to 15 serial passages in Vero cell monolayers. Sample Analysis using RT-PCR e FACS methods.

Figure 19: Physical map of recombinant pNSK
Den4/FA/Esa/EGFP plasmid with 14,498 base pairs.

Figure 20: (A) Position scheme of heterologous expression cartridge between E gene of Den4 virus and NS1 protein gene of FA virus. (B) Position of structural genes, of NS1 gene and of different domains of heterologous expression cartridge in the genome of 17D/Den4/FA/Esa/EGFP/6 virus.

Figure 21: Kinetics spreading proprieties of chemiric 17D/Den4/FA/Esa/EGFP/6 virus in Vero cell monolayers.

Figure 22: Genetic stability of 17D/Den4/FA/ Esa/EGFP/6 virus after serial seeding in Vero cell monolayers (20 passages in total).

Figure 23: Physical map of recombinant T3 Esa$_{trun}$ EGFP plasmid.

Figure 24: Analysis by fluorescence optical microscopy of Vero cell monolayers infected by 17D/Esa$_{trun}$/4$_{glic}$ and 17D/Esa/5.1$_{glic}$ viruses 72 and 96 hours after infection.

Figure 25: Regional scheme of viral genome included within prM protein and NS1 encoding genes in recombinant 17D/Esa$_{trun}$/4$_{glic}$, virus, detailing amino acid sequence of truncated stem anchor region associated to heterologous expression cartridge.

Figure 26: Kinetics graphics of Vero cell monolayer infections by 17D/Esa$_{trun}$/4$_{glic}$ virus in a 0.02 moi.

DETAILED DESCRIPTION OF THE INVENTION

Initially, important definitions are presented for the

5   perfect understanding of the scope of this invention, namely:

- **Vector virus:** virus obtainable from a cDNA template, the genomic sequence of which was modified so as to allow cloning and expression of nucleotide

10   sequences which codify proteins or parts of heterologous proteins originating from other pathogens, specifically in the intergenic region at structural E protein level and non structural NS1. This virus can be, but is not limited to, a

15   Flavivirus, especially the 17D strain amarilic virus or its offshoot. Additionally, it may be a wild virus, attenuated or genetically modified.

- **Recombining virus:** a virus that contains, inserted in its genome, specifically in the intergenic

20   region at E structural and NS1 non structural protein level, sequences of codifying nucleotides of the whole or part of heterologous proteins from other pathogens. This virus can be, though not limited to, a Flavivirus, especially the 17D strain

25   amarilic virus or its offshoot. Additionally, it can be a wild virus, atenuated or genetically modified. The recombining flaviviruses can also be chimerical viruses in which the prM/E genes of a flavivirus are replaced by homologous genes of

another flavivirus. Such viruses are useful in the development of vaccines for human and animal use, granting immune response not only in relation to Yellow fever or other virus occasioned disease, as well as in relation to diseases provoked by said other pathogens. And, in the specific case of such vaccinal application, they should be produced in embryonated hen eggs or in certified cells culture for the production of vaccines for human use (such as Vero cells, MRC-5, primary cultures of chick embryo fibroblast or others in which the recombining viruses will replicate) And, subsequently, may be utilized, in conjuction with at least one pharmaceutically acceptable vehicle, in vaccinal compositions.

- **Atenuated virus**: a virus which ability for causing an accentuated infection and, consequently, produce disease, is lesser when compared with non attenuated, or wild virus.

- **Wild virus**: a virus that can be found, or isolated from living things in their natural environment, existing in the form of laboratorial stock, whose characteristics of pathogenicity are maintained despite being kept in laboratories without intermediary passages in a natural host. This wild virus may also exist in the form of a wild recombining virus after undergoing genetic manipulation in laboratory.

- **Offshoot of 17D strain amarilic virus:** constitutes of ramifications, or substrains, of the vaccinal strain of the 17D yellow fever virus, that are obtained from this through a differentiated historic of passages in different kinds of cellular substracts permissible to viral replication. Nowadays, the vaccines for human use are derived from two distinct substrains, the 17D-204 and the 17DD.

- **Virulent forms homologous to the vector virus:** constitutes of - as virulent forms homologous to the vector virus - a more pathogenic virus, being homologous to the attenuated one and differing from same in only some positions in the viral genome. For example, in the case of the vaccinal virus of FA (17D), this one differs from the virulent wild virus, of which it derived by serial passages in culture (process through which the genetic mutations accumulated), in only 48 nucleotides in the viral genome of 10862 nucleotides (0,44% of nucleotide difference), representing only about 22 aminoacid alterations along the 3411 aminoacids of the viral polyprotein (about 0,65% of differences from the aminoacid sequence).

- **Functionally Equivalent Sequences:** sequences can be denominated equivalent if they play the same role, without being identical from the aminoacid or nucleotidic sequence viewpoint, over a considered utilization or application. The equivalent

sequences may be the result of variability, meaning, any modification, spontaneous or induced, in a sequence, be it substitution and/or deletion and/or insertion of nucleotides, and/or extension and/or shortening of the sequence at one of its ends. A non natural variability may result from genetic engineering techniques.

- **nucleotidic heterologous (or exogenouss) modified sequences**: sequences (including, but not limited to those of 10 to 2000 nucleotides) from viruses or other pathogens, which are modified before the insertion in the vector virus. Such modification is carried out so that the same, when cloned and expressed in the vector virus, possess, in its 5´ portion, nucleotides present at the 5´ end of the NS1 gene of this vector virus or of other functionally equivalent virus or sequences, and in its 3´ portion, a genome region corresponding to the whole or a part of the domains of stalk and anchor of the E protein of this vector virus or of other functionally equivalent virus or sequences.

- **Heterologous expression cartridge**: expression genic construction in viral genome or functional equivalents, structured to enable viral sequences fusion to heterologous gene to be expressed in a manner in which its expression effectiveness is improved. In this matter, EGFP gene suffers a fusion of its 5' encoding terminal edge to 27 nucleotides corresponding to NS1 protein N-terminal

and of its 3′ encoding element to the complete
genic sequence, or part of it, of the stem and
anchor domains.

This way, this invention relates to the genetic
5   manipulation of viruses, including, though not limited to,
Flavivirus, preferably the 17D strain vaccinal amarilic
virus (the sequence of which is represented by SEQ ID No
15) or its derivatives; envisaging its utilization as
heterologous antigen expression vector and the development
10   of new attenuated live vaccines.

The following method is one of the objects of this
invention, namely:

Method for the production of recombining virus
containing sequences of codifying nucleotides of whole or
15   part of heterologous proteins, characterized by the
following phases:

a) Modification of heterologous nucleotide sequences
so as the same, when cloned and expressed in the
vector virus, will possess, in their 5′ portion,
20   nucleotides present at the 5′ end of the NS1 gene
of this vector virus or of other functionally
equivalent viruses or sequences, and in their 3′
portion, a genome region corresponding to the
whole or part of the stem and anchor domains of
25   the E protein of this vector virus or of other
functionally equivalent viruses or sequences, and
so not jeopardizing the structure and the said
vector virus replication;

b) Insertion of the heterologous sequences modified

in a) in the intergenic region at structural E
protein level and of non structural NS1 of the
vector virus;

c) Obtention of recombining non pathogenic virus and
5   holder of immunologic properties, containing the
heterologous sequences stabilized integrated in
the viral genome according to insertion in the
region described in (b) and, therefore expressing
the heterologous antigen so that the same induces
10   an adequate immune response.

In an embodiment of this invention, the abovementioned
method is characterized by the fact that heterologous
nucleotide sequences are modified in (a) so that the same,
when cloned and expressed in the virus, will possess, in
15 their 5´ portion, the nucleotides described in SEQ ID No. 1
(codifiers of SEQ ID No 5) or their functionally equivalent
sequences and, in their 3´ portion, the genome region
corresponding the domains of stalk and anchor of the viral
E protein as described in SEQ ID No. 3 (codifiers of SEQ ID
20 No 7) or their functionally equivalent sequences.

However, for the development of the present method and
the consequent obtention of these recombining viruses,
especially of flavivirus, expressing heterologous antigens,
it has been necessary:

25   (a) the drawing of strategies to allow the
introduction of heterologous antigens, without
jeopardizing the structure and replication of the
vector virus;

(b) to ensure that the construction of cDNA (and its RNA transcripts) generates a non-pathogenic recombining virus and the foreign sequence, beyond that, be stably integrated in the viral genome; and

(c) to guarantee that the recombining virus resulting from the abovementioned method, besides being attenuated, will retain its immunologic properties, expressing the heterologous antigen, inserted so as the same will induce an adequate immune response (measured by the formation of antibodies against the viral and recombining proteins), directed both to the vector virus (or virulent forms homologous thereto) and to the heterologous antigen. It is also important the maintenance of the replication capability in cultures of certified cells for the production of vaccines.

In this sense, the presence of specific sequences (nucleotides present at the 5′ end of the NS1 gene and a genome region corresponding to the whole or part of the domains of stalk and anchor of the E protein) of this vector virus or of other virus, especially flavivirus, associated with protein Exogenous, envisages to minimize or eliminate potential negative effects in the viral replication in function of heterologous insertion in the E/NS1intergenic region, since:

(1) the 5′ end of the NS1 protein is part of the recognition region of the cellular signalase for

the generation of the E and NS1 proteins, so as

the Exogenous protein undergoes the same kind of

processing, not · disturbing the obtention of

protein, and allowing the heterologous protein to

5   be correctly processed by cellular signalase in

the membrane of the endoplasmic reticulum;

(2) The whole or part of the stalk and anchor domains

of the E protein, that are added to protein

Exogenous, allow normal processing of the NS1

10   viral protein to occur, given that it possesses

the sequence signal for processing by E/NS1

junction signalase.

Therefore, it is prudent to stress that the capability

of introducing genetic modifications in the animal viruses

15 has promoted knowledge on the mechanisms involved in the

viral propagation, besides allowing these to begin to be

used as heterologous proteins expression vectors. DNA

viruses – such as SV40, vaccinia, and herpes – are examples

of viral vectors for the expression of exogenous

20 insertions.

The advance in the molecular cloning tecniques has

led, more recently, to the development of RNA viruses,

positive or negative ribbon, such as viral vectors (Palese,

P. 1998. RNA vector virus: where are we and where do we

25 need to go? Proc Natl Acad Sci U S A. 95:12.750-12.752).

These are, potentially, more advantageous than the DNA

viruses, since they do not have a DNA phase and are not

capable of integration in the genome of the host.

One of the most promising positive ribbon Viral RNA vectors is the virus of the Flavivirus genus. Among these, is the yellow fever virus, for which there is the sole licensed attenuated virus vaccine against this group of

5   human pathogens.

The yellow fever vaccine is composed by 17D strain vaccinal virus. This vaccine is extremely efficient, promoting about 95 % of seroconversion and lasting imunity in the inoculated individuals; detection of neutralizing

10 antibodies being possible, even after periods of over 30 years post inoculation, as can be evidenced in a study made by Poland et al. (Poland, J. D., C. H. Calisher, T. P. Monath, W. G. Downs, and K. Murphy. 1981. Persistence of neutralizing antibody 30-35 years after immunization with

15 17D yellow fever vaccine. Bull World Health Organ 59:895-900). Additionally, the yellow fever vaccine has other attractive properties that subsidize its development as a recombining vaccinal vector, which would be:

(i) a very well defined production methodology;

20   (ii) consisting of a cheap single shot vaccine; and

(iii) its estimated use is of about 400 million shots administered, with occurrence of few cases of adverse side effects (Monath, T. P. 2001. Yellow fever: an update. Lancet Infect Dis 1:11-20).

25 Due to these good properties, the FA 17D vaccine platform is being utilized in the development of human recombining vaccines against other pathogens, for which, hitherto, no established vaccines exist, as per the example given by some diseases caused by flavivirus, like the

Japanese encephalitis (Chambers, T. J., A. Nestorowicz, P.
W. Mason, and C. M. Rice. 1999. Yellow fever/Japanese
encephalitis chimeric viruses: construction and biological
properties. J Virol 73:3095-101; Monath, T. P., F.

5 Guirakhoo, R. Nichols, S. Yoksan, R. Schrader, C. Murphy,
P. Blum, S. Woodward, K. McCarthy, D. Mathis, C. Johnson,
and P. Bedford. 2003. Chimeric live, attenuated vaccine
against Japanese encephalitis (ChimeriVax-JE): phase 2
clinical trials for safety and immunogenicity, effect of

10 vaccine dose and schedule, and memory response to challenge
with inactivated Japanese encephalitis antigen. J Infect
Dis 188:1213-30) and dengue (Guirakhoo, F., K. Pugachev, Z.
Zhang, G. Myers, I. Levenbook, K. Draper, J. Lang, S.
Ocran, F. Mitchell, M. Parsons, N. Brown, S. Brandler, C.

15 Fournier, B. Barrere, F. Rizvi, A. Travassos, R. Nichols,
D. Trent, and T. Monath. 2004. Safety and efficacy of
chimeric yellow Fever-dengue virus tetravalent vaccine
formulations in nonhuman primates. J Virol 78:4761-75),
malaria (Bonaldo, M. C., R. C. Garratt, P. S. Caufour, M.

20 S. Freire, M. M. Rodrigues, R. S. Nussenzweig, and R.
Galler. 2002. Surface expression of an immunodominant
malaria protein B cell epitope by yellow fever virus. J Mol
Biol 315:873-85; Bonaldo, M. C., R. C. Garratt, R. S.
Marchevsky, E. S. Coutinho, A. V. Jabor, L. F. Almeida, A.

25 M. Yamamura, A. S. Duarte, P. J. Oliveira, J. O. Lizeu, L.
A. Camacho, M. S. Freire, and R. Galler. 2005. Attenuation
of recombinant yellow fever 17D viruses expressing foreign
protein Epitopes at the surface. J Virol 79:8602-13; Tao,
D., G. Barba-Spaeth, U. Rai, V. Nussenzweig, C. M. Rice,

and R. S. Nussenzweig. 2005. Yellow fever 17D as a vaccine vector for microbial CTL epitopes: protection in a rodent malaria model. J Exp Med 201:201-9) and, even as could be seen in a study carried out on mice, directed towards melanoma cells (McAllister, A., A. E. Arbetman, S. Mandl, C. Pena-Rossi, and R. Andino. 2000. Recombinant yellow fever viruses are effective therapeutic vaccines for treatment of murine experimental solid tumors and pulmonary metastases. J Virol 74:9197-205).

RNA viruses are considered to have more resistance to the introduction of heterologous genes, when compared to the DNA viruses, which can be observed with the bicistronic vectors of the West Nile fever and the yellow fever virus, which contained interneal ribossomal entry sites (Patent Document WO02089840; Pierson, T. C., M. S. Diamond, A. A. Ahmed, L. E. Valentine, C. W. Davis, M. A. Samuel, S. L. Hanna, B. A. Puffer, and R. W. Doms. 2005. An infectious West Nile virus that expresses a GFP reporter gene. Virology 334:28-40). However, one should consider that these modifications were made in the 3' region not translated in the flaviviruses genome; region that, despite showing a certain variability in FA virus size (de Filippis, A. M., R. M. Nogueira, H. G. Schatzmayr, D. S. Tavares, A. V. Jabor, S. C. Diniz, J. C. Oliveira, E. Moreira, M. P. Miagostovich, E. V. Costa, and R. Galler. 2002. Outbreak of jaundice and hemorrhagic fever in the Southeast of Brazil in 2001: detection and molecular characterization of yellow fever virus. J Med Virol 68:620-7; Mutebi, J. P., R. C. Rijnbrand, H. Wang, K. D. Ryman, E.

Wang, L. D. Fulop, R. Titball, and A. D. Barrett. 2004.
Genetic relationships and evolution of genotypes of yellow
fever virus and other members of the yellow fever virus
group within the Flavivirus genus based on the 3' noncoding
region. J Virol 78:9652-65), presents itself highly
structured with regions forming much conserved secondary
structures (Holden, K. L., and E. Harris. 2004. Enhancement
of dengue virus translation: role of the 3' untranslated
region and the terminal 3' stem-loop domain. Virology
329:119-33; Thurner, C., C. Witwer, I. L. Hofacker, and P.
F. Stadler. 2004. Conserved RNA secondary structures in
Flaviviridae genomes. J Gen Virol 85:1113-24). These are
involved in the control of translation process   (Chiu, W.
W., R. M. Kinney, and T. W. Dreher. 2005. Control of
translation by the 5'- and 3'-terminal regions of the
dengue virus genome. J Virol 79:8303-15) and viral
replication (Tilgner, M., T. S. Deas, and P. Y. Shi. 2005.
The flavivirus-conserved penta-nucleotide in the 3' stem-
loop of the West Nile virus genome requires a specific
sequence and structure for RNA synthesis, but not for viral
translation. Virology 331:375-86; You, S., B. Falgout, L.
Markoff, and R. Padmanabhan. 2001. In vitro RNA synthesis
from exogenous dengue viral RNA templates requires long
range interactions between 5'- and 3'-terminal regions that
influence RNA structure. J Biol Chem 276:15581-91; Yu, L.,
and L. Markoff. 2005. The topology of bulges in the long
stem of the flavivirus 3' stem-loop is a major determinant
of RNA replication competence. J Virol 79:2309-24). The
insertion of sequences of the SIER kind, which form

secondary structures at the non translated 3' end of the viral genome, could, then, interfere with these key processes to viral variability.

In this invention, a strategy for insertion - of proteins or exogenous proteic domains - between the codifier gene of the E protein and that of NS1 protein was developed.

This insertion site represents, firstly, a vital point in the viral multiplication process. The same consists of the transition of a genic block encoding the viral proteins constituting the viral particle (C, prM and E), and the other codifying the non structural proteins, that are involved in the process of viral replication. The insertion of a heterologous sequence between these blocks could be less harmful to the cascade of molecular events that occurs in this region during replication, since it would be in a intergenic region. And, in these, in principle, there would be no need for special proximity between the two adjacent viral proteins in the recently translated polyprotein; such as for example, would be expected between the structural C, prM and E proteins. The prM and E proteins are sequentially translocated to the ER and interact, forming heterodimers, which, in turn, will take part in the viral particle. Another example would be between NS2B and NS3 proteins, where the insertion of long sequences may result in considerable removal from NS2B, cofactor of NS3, as well as the loss of proteolytic activity and inhibition of the viral polyprotein processing after its synthesis (Bonaldo, MC and Galler, R, unpublished information).

However, in order to be able to insert strange genes in this region, it is necessary to comply with certain restrictions for the viral polyprotein to be correctly processed and the virus be feasible. In the first place, the ectodomain of the E protein is bound to the cell membrane, or to that of the viral envelope, by means of a region called stalk and anchor. This region is conserved between the different members of flaviviruses, indicating an important function (Cocquerel, L., C. Wychowski, F. Minner, F. Penin, and J. Dubuisson. 2000. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins. J Virol 74:3623-33; Stiasny, K., S. L. Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142-7). Such sequence is constituted by 96 aminoacid residues of the C-terminal end of the protein (Allison, S. L., K. Stiasny, K. Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope E protein. J Virol 73:5605-12). The stalk domain is composed of two potential alfa-helixes (H1 and H2) connected by a sequence highly conserved in flavivirus (CS), the function of which has not been established yet. The H1 segment appears to be involved in the process of conversion of monomers into trimers during the merger of the viral envelope to the endossome membrane. The second

amphipathic element of the stalk (H2), along with the first transmembrane domain (TM1), are important for the prM/E dimer stability. The second TM2 stretch works as a signal sequence for the importation of NS1 for the ER. This way,

5   the E protein is anchored inside the ER lumen, through two transmembrane domains, TM1 and TM2, which promote its association to the lipid bilayer. During the process of translocation of the E protein to the ER, TM1 has the function of stopping the transference of E protein to the

10 ER lumen, besides the association to the ER membrane. TM2 consists of a signal sequence, which promotes, in its turn, the translocation of the NS1 to the ER lumen. The role of each of these different stalk and anchor components of the E protein has not been elucidated yet; but, for the correct

15 topology of the E protein in the ER membrane, two sequences equal or functionally similar to the anchor TM1 and TM2 sequences are needed. TM2 works as a signal peptide, which, when processed by the signalase, results in the formation of the protein carboxi-terminal and, besides promoting the

20 translocation of the NS1 protein to the ER.

For these reasons, initially, the attempt for coning and expression of the EGFP autofluorescent protein gene – a variant of the "Green Fluorescent Protein" or GFP of *Aquorea Victoria* (Cormack, B. P., R. H. Valdivia, and S.

25 Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173:33-8) – was traced in function of outflanking this exogenous gene through these sequences. In this way, no considerable disturb is provoked in the cellular addressing and proteolytic processing of E

and NS1 proteins. Another important aspect in this wise relates to the existence of the correct sequence to be cleaved, by the peptidase signal, in the junction between the TM2 anchor sequence and the NS1 N-terminal. One may

5   notice, in Figure 6, that the site - around the peptide bond hydrolysis point, for the generation of the C-terminal ends of the E protein, and of N-terminal end of the NS1 protein - is much preserved between different flavivirus. This fact indicates that the same should be important for

10 the recognition and promotion of the proteolysis site specified by the signal peptidase at the E/NS1 junction.

Figure 6 is associated to the cleavage point of the signal peptidase in the E and NS1 intergenic region of flaviviruses. In (A), alignment of the last seven residues

15 of the E protein C-terminal and the nine initial residues of the NS1 protein N-terminal around the cleavage point through the cellular signalase. In (B), consensus motive around hydrolysis point of the peptide bond (↓). The sequences utilized in the alignment are: TBE virus (Genbank

20 NC 001672), yellow fever virus (Genbank U17066), japanese encephalitis virus (JE; NC001437), west nile fever (WN; NC001563), dengue 2 (Den 2; NC001474) and dengue 4 (Den4; M14931). Residues conserved between the species are indicated by grey shading. X means lack of conservation at

25 position. The sequence alignment was carried out through the CLUSTAL W(1.82) program, which consists of a method for progressive alignment of multiple sequences. This analysis was done at http://www.ebi.ac.uk/clustalw/index.html.

So, for the correct processing, both of E protein C-terminal and of NS1 protein N-terminal, it is necessary that the Exogenous protein presents, in its N-terminal, an aminoacid sequence of the NS1 N-terminal and, in its C-

5 terminal, a corresponding E protein C-terminal aminoacid sequence.

Therefore, this invention is associated to the methodology of inserting heterologous sequences between the structural and non structural viral genes (including,

10 though not limited to, Flavivirus, preferably the 17D vaccinal strain amarilic virus or its offshoot), through the strategy of translocation and anchoring in several cellular compartments of the heterologous proteins through the genetic merger with the regions called stalk and anchor

15 of any virus or of functionally equivalent sequences.

In a preferential embodiment of this invention the amarilic virus is employed as vector virus. Therefore, once the amarilic virus genome is made of ARN, in this invention, any manipulation thereof is made at

20 complementary ADN (cADN) level cloned in bacterial plasmids. This manipulation is carried out through the infectious clone technology, which consists in the ability of regenerating viruses from cloned complementary ADN.

This invention is thoroughly described through the

25 examples shown below. It is necessary to stress that the invention is not limited to these examples, but also includes variations and modifications within the limits in which it works.

EXAMPLE 1: Drawing of the EGFP protein expression cassette in the intergenic region.

The EGFP gene and aminoacids sequence is presented, respectively, in SEQ ID No. 2. and in SEQ ID No. 6.

5   One of the possible theoretic drawings of the cloning and expression of an Exogenous protein in the intergenic region - between the coding genes for the E and NS1 proteins - consists of the genomic insertion of this heterologous sequence, outflanked by genomic flavivirus

10   sequences duplicated in this construction; in such a way that this will not disturb the translocation and cellular location of the E and NS1 proteins. In this sense, the strategy used was that of building the insertion so that, at its coding 5' end, the 27 nucleotides corresponding to

15   the NS1 protein N-terminal were merged and, at its 3' end, the gene region corresponding to E protein C-terminal stalk and anchor domains (Figure 7). Thus, with these duplicated flavivirus genome regions outflanking the insert, there are conditions for adequate processing of the E protein

20   anchored in the ER membrane - in that case, with the presence of the TM2 domain (which is a signal sequence) and part of the NS1 amino end, which allows the addressing to the ER and the specific site cleavage through the ER membrane signal peptidase. This results in the formation of

25   E protein C-terminal and the recombining protein amino-terminal release in the ER lumen. Additionally, the merger of stalk domain and anchor to the exogenous protein C-terminal, promotes its anchoring to the ER membrane; besides rendering possible that NS1 protein be translocated

to the ER lumen, due to the presence of the inner signal
peptide in the TM2 domain.

Figure 7 is associated to a comparison of E and EGFP
protein topology - cloned and expressed, in a recombining
5   flavivirus, in the intergenic region between the E and NS1
proteins in ER membrane. In (A), the membrane topology
expected for the E protein in a cell infected by a non
recombining flavivirus is presented. The black arrow
indicates o ponto de proteolytic processing, through the
10   signalase, for the formation of the carboxi terminal of
this protein and the NS1 protein amino terminal. In (B),
the expression in the recombining viruses of the EGFP
protein inserted between the E and NS1 proteins. The EGFP
protein is fusioned, in its amino-terminal with 9 residues
15   of the NS1 protein amino-terminal - SEQ ID No. 5 (black
line), and the cellular signalase cleaves at the indicated
point (black arrow). In this manner, there is formation of
the E protein C-terminal anchored to the membrane,
releasing the amino-terminal of the NS1/EGFP merger in the
20   ER lumen. This very processing would be carried out in the
C-terminal region of the stalk domain anchor fusioned to
EGFP, which would promote the association of the EGFP to
the ER membrane and the liberation of NS1 protein to the ER
lumen. The foreseen sequence of this expression cassette
25   contained in the viral polyprotein is presented in the SEQ
ID No. 14, as well as, the expected aminoacid sequence of
the recombining protein after the phases of proteolytic
processing (SEQ ID No. 8).

In the E protein homologous of the yellow fever virus, the establishment of the regions corresponding to stalk and anchor conserved domains, previously elucidated for the E protein of the TBE virus (Allison, S. L., K. Stiasny, K.

5   Stadler, C. W. Mandl, and F. X. Heinz. 1999. Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope E protein. J Virol 73:5605-12, Stiasny, K., S. L. Allison, A. Marchler-Bauer, C. Kunz, and F. X. Heinz. 1996. Structural requirements for low-pH-

10 induced rearrangements in the envelope glycoprotein of tick-borne encephalitis virus. J Virol 70:8142-7), was effected through the alignment of C-terminal residues of both proteins (Figure 8A). After this alignment, the regions corresponding to different stalk domain segments

15 (H1, CS and H2) and anchor (TM1 and TM2) were located in the sequence of Yellow fever virus E protein residues. This alignment allowed definition of the aminoacids sequence segments to be added to EGFP protein in accordance with the established strategy. A copy of this entire region for the

20 yellow fever virus, consisting of 288 nucleotides (SEQ ID No. 3) corresponding to 96 E protein final carboxi aminoacids residues (SEQ ID No. 7), was fusioned to the codifying sequence of the EGFP autofluorescent reporter protein in its corresponding C-terminal end, so as to

25 reproduce all motives contained in this sequence, and which are necessary for the correct addressing and processing of the NS1 protein, located later.

A second additional type of aminoacid sequence, derived from the yellow fever virus genome, was associated

to the EGFP protein N-terminal. This sequence represents
the 9 residues of NS1 protein N-terminal (Figure 9B), which
are also presented by SEQ ID No. 5. Three out of four
aminoacids of this peptide amino-terminal are highly
5 conserved among the flavivirus. In all likelihood, they are
important for recognition, and bond to the active center
and   proteolitic   cleavage   through   signal   peptidase
associated to ER membrane. The use of this sequence, merged
to the heterologous protein N-terminal portion, helps
10 promoting   the   correct   cleavage   between   this   and   the   E
protein, so as to form the mature E protein C-terminal and
the EGFP protein N-terminal. The utilization of part of NS1
protein N-terminal was already reported, in plasmids of
expression of prM and E genes, for the production of
15 subviral particles of TBE in cultures of eucaryote cells,
as described by Allison et al. (Allison, S. L., C. W.
Mandl, C. Kunz, and F. X. Heinz. 1994. Expression of cloned
envelope protein genes from the flavivirus tick-borne
encephalitis   virus   in   mammalian   cells   and   random
20 mutagenesis by PCR. Virus Genes 8:187-98). In the work of
these researchers, the first 30 codes (120 nt) of the NS1
protein gene were utilized - a sequence considerably
greater than that utilized in this construction, with the
equivalent to 9 codons of the first yellow fever virus NS1
25 protein N-terminal aminoacid residues (SEQ ID No. 5).

This way, the clonage of this kind of EGFP expression
cassette,   or   other   exogenous   protein,   in   the   E/NS1
intergenic region should promote the release of this
protein amino terminal in the ER lumen and the anchoring of

its carboxi end to the ER membrane, through the stalk and
anchor domains or functionally equivalent sequences.

In Figure 8, regions of E and NS1 protein used in the
assembly of the EGFP protein expression cassette in the
infectious clone FA 17D are presented. Particularly, Figure
8(A) is relative to the stalk and anchor domain of the
Yellow fever virus E protein; as well as the alignment of
the aminoacid sequence of TBE virus E protein stalk and
anchor domains (residues of 401 to 496; Genbank NC 001672)
and of yellow fever virus (residues of 398 to 493; Genbank
U17066). The residues conserved between species are
indicated by *, with conservative substitution for or less
conservative for. (B) Alignment of the nine residues of NS1
protein amino-terminal end of different flaviviruses. The
residues conserved are highlighted in grey in the different
viral sequences. The sequences used in the alignment are,
in part, the ones described in section (A). The remaining
ones are those described for the virus of Japanese
encephalitis (JE; NC001437), west of Nile fever (WN;
NC001563), dengue 2 (Den 2; NC001474) and dengue 4 (Den4;
M14931). The alignment of multiple sequences was made
through the method of CLUSTAL W, available at
http://www.ebi.ac.uk/cgi-bin/clustalw/.

EXAMPLE 2: Synthesis and cloning of the EGFP expression
cassette.

For obtention of an EGFP protein expression cassette,
two DNA fragments were initially synthesized by PCR:

(1) a DNA fragment of 783 pb containing the EGFP gene,
utilizing the pEGFP-C2 plasmid (BD Biosciences Clontech)

and the synthetic RG 328 (SEQ ID No. 9) and RG 329 (SEQ ID No. 10) oligonucleotides. The RG 328 (SEQ ID No. 9), of positive polarity, contained seqüencially the gene regions of 15 nucleotides corresponding to the protein carboxi-terminal and, 27 nucleotides corresponding to the first nine aminoacids of the NS1 protein; beyond the 20 nucleotides of the EGFP gene 5´terminal. The RG 329 (SEQ ID No. 10), of negative polarity, contains sequentially the gene regions of 24 nucleotides of the EGFP gene 3´terminal, 15 nucleotides corresponding to the E protein stalk and anchor domains N-terminal;

(2) A second fragment de 339 pb was obtained, utilizing the T3 plasmid and the RG 330 (SEQ ID No. 11) and RG 331 (SEQ ID No. 12) synthetic oligonucleotides, so as to obtain a DNA fragment containing: from sense 5´to 3´ of the coding ribbon, the 24 nucleotides corresponding to the EGFP protein carboxi-terminal, followed by gene region of 288 nucleotides (SEQ ID No. 3), corresponding to E protein stalk and anchor domains (genome position FA of 2165 to 2452); followed, finally, by the gene region of 27 nucleotides, corresponding to 9 residues of the amino-terminal of NS1 protein (genome position FA of 2453 to 2479) as described in SEQ ID No. 5.

The merger of these two DNA fragments, for the generation of the EGFP protein expression cassette to be cloned yellow fever virus genome, was carried out by reaction of PCR with equimolar quantities of the de 783 pb and 339 pb fragments, in the presence of 20 µM RG 328 (SEQ ID No. 9) and of RG 331 (SEQ ID No. 12). All those PCR

reactions were made with the Platinum *Pfx* Polymerase enzyme
(Invitrogen), in accordance with the manufacturer's
recommendations. The reaction products were analyzed in
agarose gel electroforesis at 1% and purified,
5   subsequently, by PCR (Qiagen) products purification system.
Figure 9B shows the expected product sequence, that is
decurrent from this sequence of viral origin association
strategy at the amino- ends and EGFP protein carboxi-
terminal.

10   The fragment resulting from 1071 pb was cloned in the
pGEM-T(Promega) plasmid, in accordance with the
manufacturer's specifications. Component bacteriae *E. coli*
MC1061 were transformed with 10 ng of the bond and plaqued
in selective means (LB a 1,5 % agar containing 50 µg/mL
15   ampicilin). Preparations of recombining bacterial clones
plasmidial DNA were obtained and submitted to digestion
with a Nar I enzyme, for confirmation of cloning of the DNA
cassete of 1029 pb (SEQ ID No. 4). One of the bacterial
clones was chosen, and the plasmidial DNA was purified as
20   described in one of the following sections.

Therefore, Figure 9B shows an aminoacid sequence, that
is predicted for the heterologous insertion, and that
contains o EGFP gene cloned in E/NS1 intergenic region. (A)
Aminoacid sequence in the intergenic region between the TM2
25   domain of the E protein and o NS1 protein N-terminal. (B)
This same intergenic region containing the insertion of the
heterologous expression cassette. The gray arrows indicate
the cleavage site through the signal peptidase associated
to the ER membrane.

About 10 μg of the pGEM-T plasmid, containing the EGFP protein expression cassette, was digested with 3U of Nar I (Promega). The sample was concentrated by precipitation with etanol and ressuspended in electroforesis sample
5   buffer, besides being submitted to electroforesis in agarose gel at 1%. The DNA strand of 1029 pb (SEQ ID No. 4) was purified from the gel through the DNA purification system from agarose gels (Qiagen). The material was quantified by espectrophotometry at 260 nm and analyzed in
10   agarose gel electroforesis at 1%.

The DNA fragment of about 1 kb, containing the cohesive Nar I ends, was bound to the vector T3 plasmid. This plasmid is a derivate from the original pYFM5.2, containing the 17D genome central region, and which
15   contains a restriction site of Nar I just at the junction between the coding genes for the E and NS1 protein. The bond was made with the T3 plasmid, previously digested with Nar I, in the presence of a molar excess 20 times of the insertion containing the EGFP gene, and of the T4 DNA
20   ligase enzyme (Invitrogene). The corresponding to 10 ng of the bond was transformed into *E.coli* Sure (Stratagene), which was plaqued in selective means LB 1,5% agar containing 50 μg/mL of ampicilin. Mini preparations of plasmidial DNA were made, from the ampicilin resistant
25   bacterial colonies; and the plasmidial DNA preparations, that presented size superior to that of pT3 native control, were submitted to digestion with Nar I for confirmation of the cassete cloning. The verification of the correct sense of insertion insertion was carried out by nucleotidic

sequencing. This way, the recombining pT3 Esa EGFP plasmid
was obtained, as in Figure 10.

In Figure 10, the physical map of the T3 Esa EGFP
recombining plasmid is presented. The original pT3 plasmid,
5   that contains part of the cloned viral cDNA (from the
genome position of 1373 to 9428), was used for cloning EGFP
protein expression cassette in the Nar I site of insertion.
This recombining plasmid was, afterwards, used for
assembling the viral cDNA template.

10   **EXAMPLE 3**: Preparation of the cDNA viral template.

The cDNA template, utilized in the obtention of the FA
17D recombining virus, was obtained by the two-plasmid
system (Rice, C. M., A. Grakoui, R. Galler, and T. J.
Chambers. 1989. Transcription of infectious yellow fever
15   RNA from full-length cDNA templates produced by in vitro
ligation. New Biol 1:285-96; Patent Document US 6.171.854).
In this, the original plasmids, pYF5'3'IV - that contain
part of the cloned genome in the form of cDNA (the 5´ ends,
position of 1 to 2.276, and 3', position of 8.275 to
20   10.862) - and pYFM5.2 - containing the central genomic
portion (nt of 1.373 to 9428)- are used for the assembly of
complete viral cDNA, by means of a series of cutting enzyme
reactions and DNA fragments bond. In the creation of EGFP
expression cassette, a derivate of pYF5'3'IV was used,
25   called pE200$_{glic}$, which presents mutations in the 1568
nucleotid, that result in the criation of an *EcoRV* site in
the position of the E protein 200 aminiacid. Such fact
leads to change of two aminoacids (E199 D and T200I), as
described by Bonaldo et al. (Bonaldo, M. C., R. C. Garratt,

P. S. Caufour, M. S. Freire, M. M. Rodrigues, R. S. Nussenzweig, and R. Galler. 2002. Surface expression of an immunodominant malaria protein B cell epitope by yellow fever virus. J Mol Biol 315:873-85), and the presence of

5   the N-glicosilation motive in the protein and, in 1436 and 1437 genome positions. The second plasmid, in which exogenous protein expression cassette was cloned, was a plasmid derived from pYFM5.2, called pT3/Esa/EGFP plasmid. The template of viral cDNA was prepared by cleavage of the

10 plasmids with the Nsi I and Sal I (Promega) restriction enzymes, in complianc with the conditions reccommended by the manufacturer. About en µg of each plasmid were digested with both enzymes. The cleavage was monitored by the analysis of percentages equivalent to 200 ng of DNA in

15 agarose gel electroforesis at 0,8% in buffer TAE. Upon complete cleavage, the enzymes were inactivated by heat. The NSiI/SalI cleavage products of the plasmids were bound with T4 DNA ligase (Epicentre Technologies) in compliance with the conditions set forth by the manufacturer. The

20 linearization of the different cDNA templates was done by use of Xho I restriction endonuclease under the conditions established by the manufacturer (Promega). The resulting products were precipitated with ethanol and ressuspended in Tris-EDTA buffer, pH 7,5, free of nucleases. A sample of

25 each preparation was analyzed in agarose gel electroforesis for detection of the template and its quantification. The preparations were stored at -20°C until the phase of transcription in vitro.

Obtention of FA virus from viral cDNA: transcription and transfection phases.

From the cDNA templates representing the complete genome, including the sequences of the $pE200_{glic}$ and
5   pT3/Esa/EGFP plasmids, preparations of viral RNA were obtained through the transcription system *in vitro* of SP6 RNA (AmpliScribe SP6; Epicentre Technologies). The synthesized preparations of RNA *in vitro* were analyzed in electroforesis in gel of agarose 0,8% in TAE. Percentages
10   of the RNA preparations were transfected with Lipofectamine (Invitrogen Life Sciences) in Vero cells monolayers, as described by Bonaldo et al. (Bonaldo, M. C., R. C. Garratt, P. S. Caufour, M. S. Freire, M. M. Rodrigues, R. S. Nussenzweig, and R. Galler. 2002. Surface expression of an
15   immunodominant malaria protein B cell epitope by yellow fever virus. J Mol Biol 315:873-85).

Transfection of Viral RNA synthesized *in vitro*

The phase of transfection was carried out in a way similar to that described in patent Document US 6,171,854.
20   The transfection of the Viral RNA synthesized *in* vitro originated a recombining virus, capable of growth in Vero cells. This new recombining yellow fever virus was called $17D/Esa/5.1_{glic}$. Its detection was carried out by the appearance of cytopathic effect in the cellular monolayer
25   through phase contrast microscopy. The kinetic follow up of the EGFP protein expression was carried out in the time intervals of 24, 48, 72, 96 and 120 hours in Vero cells monolayers infected with the 17D /Esa/5.1_{glic} with virus a m.o.i of 0,02, and through fluorescence microscopy at 488

nm for detection of the EGFP autofluorescent protein expression.

In order to determine the EGFP expression kinetics, Vero cells were infected with recombining Viruses
5   expressing EGFP at a 0,02 MOI. In the different times, the cellular monolayers were washed twice with PBS, fixad with 4% paraformaldehyd in 0,1M dibasic phosphate buffer for 10 minutes, and washed once with 0,2M dibasic phosphate buffer. Upon fixation, the cells were dyed for 5 minutes
10   with Evans Blue 1%, mounted on blades – with use of Slow Fade containing DAPI (Slow Fade Gold reagent with DAPI – Molecular Probes) – and observed through a Zeiss fluorescence confocal microscope.

Figure 11 shows the kinetics de Vero cells monolayer
15   infection by 17D /Esa/5.1$_{glic}$ virus. Preparations of 24 h, 48 h, 72 h, 96 h and 120 h post-infection were analyzed. The green fluorescent marking indicates the presence of EGFP Exogenous protein, this associated in the main to the cellular ER. For comparison, one of the preparations of the
20   control condition was placed, cells not infected, that consist in the time of 96 hours post-infection (Figure 11).

A viral stock was prepared, by infecting Vero cells monolayers with the pos-transfection supernatant in a m.o.i of 0,1. This stock showed a title of 6,0 log10 PFU;mL and
25   was used in all phases of viral characterization.

Figure 12 presents the comparative diagram of the genome regions comprised between the prM and NS1 proteins of the 17D vaccinal virus (A), and of the recombining

17D/Esa/5.1$_{glic}$ (B). The genomic sequence of the 17D virus/Esa/5.1$_{glic}$ is shown in SEQ ID No. 13.

EXAMPLE **4:** Characteristic of viral propagation: determination of the viral growth kinetics and phenotype of

5 lyze plaque in Vero cells monolayers.

The growth capability of the recombining FA virus obtained was analyzed, in comparison with the FA vaccinal 17DD and 17D/14 viruses, through infection in Vero cells monolayers. Three independent experiments were carried out

10 on viral propagation kinetics in Vero cell monolayers (62.500 cells/cm^2), in a number (m.o.i) of infection of 0,02. Percentages of the cellular supernatant of the post-infection times (p.i.) of 24 h, 48 h, 72 h, 96 h, 120 h and 144 h were collected and titled.

15 In these experiments, two FA 17D viruses of vaccinal phenotype were used as virus controls. The FA17D/14 experimental vaccinal virus was obtained from a cDNA template with a sequence of the 17D/204 sublineage, in which some genetic modifications were introduced based on

20 the 17DD sublineage sequence (Patent Document US 6.171.854). The FA17D/14 virus has great lyze plaque and growth properties resembling the 17DD vaccinal virus. The second virus is a 17DD strain vaccinal stock, that is the strain utilized in the production of the yellow fever

25 vaccine in Brazil, that also has great plaque phenotype.

It can be verified that both experimental vaccinal viruses – 17D/14 and 17DD – present viral growth peaks at 72 hours post-infection, with values of 7,08 and 6,97 log10 PFU/mL, respectively. On comparing the kinetic profiles of

these two viruses with the recombining 17D/Esa/5.1glic virus, it can be noted that this shows a less pronounced growth than the two vaccinal ones, that possess very similar growth profiles in Vero cells monolayers. However,

5   the recombining 17D/Esa/5.1$_{glic}$ virus presents a viral growth peak of 6,63 log10PFU/mL in 120 hours.

Despite the recombining 17D/Esa/5.1$_{glic}$ virus showing lesser propagation potential in Vero cells monolayers, the titles obtained are still adequate for the vaccinal

10   production scale.

Figure 13. shows the replication capability of the recombining 17D/Esa/5.1glic FA virus, in comparison with 17D/14 and 17DD vaccinal, in Vero cells monolayers. These cells are being used in the production of vaccines for

15   human use (Montagnon, B.J., J.C. Vincent-Falquet. 1998. Experience with the Vero cell line. Dev Biol Stand. 93:119-223; Handa R., S. Teo, R. Booy. 2004. Influenza: current evidence and informed predictions. Expert Rev Vaccines. 2004 3(4):443-451; Monath, T.P., J.R. Caldwell, W. Mundt,

20   J. Fusco, C.S. Johnson, M. Buller, J. Liu, B. Gardner, G. Downing, P.S. Blum, T. Kemp, R. Nichols, R. Weltzin. 2004. ACAM2000 clonal Vero cell culture vaccinia virus (New York City Board of Health strain)-a second-generation smallpox vaccine for biological defense. Int J Infect Dis. 8 Suppl

25   2:S31-44).

EXAMPLE 5: Determination of the lyze plaque phenotype.

The morfologic determination of the viruses lyze plaque was made by plaqueing in Vero cells monolayers , grown at 62.500 cells/ cm^2 in 6 well plaques with a

coverage of 3 mL of 0,5% agarose of low melting point (Promega) in 199 mean suplemented with 5 % bovine fetal serum. In this experiment, two FA 17D viruses of vaccinal phenotype were used as virus controls. The FA17D/E200 virus

5   was created and recovered from an infectious clone containing mutations in the 1568 nucleotid, creating a *EcoRV* site in the 200 aminiacid protein position and, that leads to the change of two aminoacids (E199 D and T200I), which presents an intermediate plaque phenotype, as

10   described by Bonaldo et al. (Bonaldo, M. C., R. C. Garratt, P.S. Caufour, M.S. Freire, M.M. Rodrigues, R.S. Nussenzweig, and R. Galler. 2002. Surface expression of an immunodominant malaria protein B cell epitope by yellow fever virus. J Mol Biol 315:873-885). It was also utilized

15   as large lyze plaque control, the 17D /14 virus, which was described above. For visualizing the lyze plaques a solution of 10% formaldehyde was added for fixation and a subsequent dying in 0,01% violet crystal. The values assessed were obtained through the two independent

20   experiments, in which about 20 plaques/viruses/experiment were measured. The values determined are shown in Table 1.

Table 1. Phenotypic Analysis of the lyze plaque size of the virus in relation to two different experimental vaccinal viruses, 17D/14 and 17D/E200.

virus	lysis plaque diameter (mm)	
	average	deviation
17D/14	2,80	0,67
17D/E200	1,65	0,33
17D/Esa/5.1$_{glic}$	0,99	0,24

EXAMPLE 5: Analysis of the EGFP Exogenous protein expression by flow cytometry of Vero cells infected by the 17D/Esa/5.1$_{glic}$ virus.

Along the viral infection, the EGFP autofluorescent protein expression in monolayers of Vero cells was measured by flow cytometry in FACScalibur equipment (Becton Dickison; 15 mW argon laser, 488 nm) with a FL-1 filter, through analysis of 10.000 events by sample. The cells were infected in a moi of 0,02 and were prepared in the post-infection times of 24 h, 48 h, 72 h, 96 h and 120 h post-infection. Vero cells were removed from cellular monolayer by trypsinization, after washing of monolayer with PBS. The cells were ressuspended and washed twice in PBS suplemented with 4 mg/mL BSA, counted and adjusted for the density of 2,0 x 10^5 cells /mL in 1% paraformaldehyd for subsequent analysis by cytometry.

In Figure 14, it can be observed that the expression of EGFP is specific of the Vero cells infected with 17D

/Esa/5.1$_{glic}$ virus, and that its detection is greater in the times of 72 to 120 hours of infection. The bar of 41,2 % shows, in Figure 14B, the percentage of cells expressing EGFP with 120 hours of infection. These results prove that

5 the recombining 17D/Esa/5.1$_{glic}$ virus is capable of promoting a significant expression of the heterologous protein, even in cellular monolayers infected with a reduced moi, and that the maximal points of the expression are detected from 72 hours of incubation. The use of a

10 reduced moi, together with the high percentage of fluorescent cells, warrant the virus capability for replicating and disseminating to the adjacent cells.

Figure 14 shows the analysis of the EGFP fluorescent protein expression kinetics through recombining

15 17D/Esa/5.1glic virus, in Vero cells and by flow cytometry. (A) Vero cells infected by yellow fever virus, 17D/E200T3 control, that do not express the EGFP protein. (B) yellow fever recombining 17D/Esa/5.1$_{glic}$ T3 virus, that expresses the EGFP autofluorescent Exogenous protein cloned in the

20 E/NS1 intergenic region.

EXAMPLE 6: Determination of the recombining 17D/Esa/5.1$_{glic}$ virus attenuation in mice.

As a first step towards proving that the recombining 17D/Esa/5.1$_{glic}$ virus does not overstep the 17D vaccinal

25 virus, in relation to the phenotypic characteristic of neurovirulence, tests were carried out in mice.

In these, groups of 10 Swiss Webster mice (three weeks old) were inoculated, through intracerebral via, with 3.0 log$_{10}$ PFU of the 17DD vaccinal control and the other

viruses. The viral inoculative, estimated in 1.000 PFU for 30 µL, is assessed by titling in Vero cells monolayers for determinatin of the viral dose, and the animals are followed up for 21 days. The results, contained in Table 2,

5   represent the average of 3 to 5 independent experiments, depending on the viral sample.

Table 2 Study of the viral attenuation by neurovirulence test in four week old Swiss Webster mice.

	17DD	17D/E200$_{glic}$	17D/Esa/5.1$_{glic}$	medium
Death rate (%)	98,0	85,0	0,0	0,0
Average survival time (days)	11,2 ± 0,55	11,8 ± 0,64	>21	>21
Average Dose administered (PFU)	1090 ± 392	797 ± 592	802 ± 265	-

As can be evidenced in Table 2, the 17D yellow fever

10 recombining virus, expressing an EGFP heterologous protein in the E/NS1(17D/Esa/5.1glic) intergenic region, presents itself more atenuated when compared to the 17DD controls and parental 17D/E200$_{glic}$ virus. The 17DD vaccinal virus promoted 98% of mortality in the inoculated animals - with

15 average time of 11,2 days : survival - and the parental 17D/E200$_{glic}$ virus, 85,0% over an average survival time of 11,8 days, the intracerebral inoculation with the

recombining 17D/Esa/5.1glic virus does not result in death in the 21 days of observation.

These results indicate that the alterations prompted by cloning and expression of EGFP modified protein, of about 400 aminoacid residues, provoke an increase I the degree of viral attenuation.

EXAMPLE 7: Study of the recombining 17D/Esa/5.1$_{glic}$ virus immunogenicity.

The immunogenicity of the 17D /Esa/5.1$_{glic}$ virus was assessed in mice. A group of four week old BABL/c mice were immunized with about .2 doses of 50.000 PFU, administered by sub-cutaneous via, in the plantar pad at 15 day intervals. Thirty days after the last dose, blood samples from the mice were obtained by intra-orbital bleeding. The humoral immune response of neutralizing antibodies, directed to the 17D yellow fever virus, was assessed by the test essay of viral neutralization by plaqueing reduction in Vero cells monolayers (PRNT in English, "Plaque Reduction Neutralization Test"). The titles of neutralizing antibodies are given in function of greater seric dilution capable of inhibiting 50% of the lyze plaques number.

As can be verified at Table 3, the FA 17D recombining viruses were able to induce response for specific neutralizing antibodies at indexes comparable to the 17DD vaccinal virus. The seroconversion for the FA virus took place in 100% of the animals that were inoculated with the recombining 17D/Esa/5.1$_{glic}$ T3 virus. And, this immunization regime resulted in title of neutralizing antibodies, directed to the yellow fever virus, from 1:65 to > 1:520,

which are in a range comparable to that determined for the
17DD vaccinal control virus, of 1:85 - >1:1.260.

Table 3. Immunogenicity of 17D /E200$_{glic}$T3 virus in BALB/c
mice.

condition	Number of animals	% seroconversion	Answer range (PRNT) *	PRNT* average	average dose (PFU)
control	5	0	< 1:20	< 1:20	-
17DD	10	90	1:85 - >1:1.260	> 1:250	65.375
17D/E200 glic T3	10	100	1:100 - 1:325	1:200	70917
17D/ Esa/ 5.1 Glic	15	100	1:65 - >1:520	1:200	18.250

5   *Reciprocal value of the major dilution of the immunized
animal serum with each virus that should have resulted in
50% of lyze plaque inhibition.

30 days after the last shot, these animals and another
independent experimental set, vaccined with the same dose
10 regime, were challenged by intracerebral inoculation with
an average dose of 6.000 PFU of the 17DD yellow fever
vaccinal virus. Figure 15 shows the mean protection values
issued from two immunization and challenge essays. The
animals were followed up for 21 days, for notification of
15 deaths and days of occurrence. It can be verified, in the
Figure 15, that the 17D/E200glicT3 parental virus and the
recombining 17D/Esa/5.1$_{glic}$T3 promote the protection of 60

and 50%, respectively, of the animals challenged by intracerebral via though 17DD vaccinal virus (with inoculative average of 6.000 PFU), while the vaccinal virus presents a protection rate of about 90%.

5   Figure 15 shows the degree of protection afforded by the immunization of BABL/c mice with the 17D /Esa/5.1$_{glic}$T3 virus, in the faced of the challenge for intracerebral inoculation with 6.000 PFU of the 17DD vaccinal strain yellow fever virus. In the upper part of the Figure, the

10   histogram with the death rates in the challenge of animals immunized with vaccinal phenotype virus (17DD and E200$_{glic}$T3), through the virus test (17D/Esa/5.1$_{glic}$T3) and through the negative control (immunization with culture mean) is shown. In the lower part of the Figure, the values

15   obtained by the group relative to the death percentage, the average time of sobrevida, number of animals per group pf analysis and the average dose used in the vaccination regime are shown.

Example 9. Genetic Stability of virus 17D/Esa/5.1glic

20   The genetic stability of 17D/Esa/5.1glic virus insertion was assessed by two series of ten independent passages through Vero cell monolayers. Thus, when *in vitro* synthesized viral RNA was obtained, as described in example 3, it was transfected into Vero cell monolayers producing

25   recombinant virus particles. This preparation was named as first cell passage sample or 1P, and it was then used to infect Vero cell monolayers in 175 cm^2-T bottles to create a virus sample batch which was employed in most of the performed analysis with 17D/Esa/5.1 glic virus. After

cytopathic effect appeared, the viral supernatant, named as
second cell monolayer passage or 2P, was measured and store
at -70°C. It was assessed a 2P-sample titration, as well
as, in order to verify if the insertion was completed in a

5 heterologous manner, it was conducted a viral RNA
extraction of this preparation by the LS Trizol-based
method (Invitrogen, Life Technologies), and then the RT-PCR
procedure, using M-MLV enzyme (Promega Corporation) to
allow cDNA synthesis to take place in simple strips and PCR

10 reaction of Tag polymerase enzyme (Promega Corporation), as
specified by the manufacturer. In the PCR reaction
performed in plasmid DNA samples, Tag polymerase enzyme
(Promega Corporation) was also used, according to the
manufacturer specifications. RG 174 oligonucleotides (SEQ

15 ID 16) was used, in a positive and corresponding direction
to 1639 to 1659 FA genomic region, and RG 19
oligonucleotides (SEQ ID 17), in a negative and
corresponding direction to 2619 to 2639 genomic region in
order to obtain a DNA fragment with 2030 base pair (bp)

20 intended length, which includes all heterologous region.
Thus, PCR products were obtained from T3 and T3 Esa EGFP
plasmid DNA, and RT-PCR products from RNA virus
preparations were analyzed in 1% agarose gel medium with
EDTA-acetate buffer.

25   The yielding of different size products, in PCR
experiments conducted in samples of T3 Esa EGFP plasmid and
17D/Esa 5.1 glic virus samples can be explained by the
presence of direct replications of 288 nucleotides
corresponding to gene regions of stem and anchor domains.

This bidirectional synthesis of the PCR reaction is promoted by positive-strip RG 174 oligonucleotides (SEQ ID 16) alignment, which supplements the region with approximately 800 nucleotides before the 5' initial position of heterologous EGFP cartridge expression (NS1 N-terminal, EGFP gene and E-protein stem and anchor domains) and by negative-strip RG 19 (SEQ ID 17) which aligns, in the back encoding region of NS1 protein, 187 nucleotides after the end of such cartridge. It may occurs, after this alignment step during PCR reaction, that the stem and anchor gene region of this heterologous cartridge combines with the homology region, located at the supplementary negative strip, corresponding to the stem and anchor gene region of E protein (Figure 16C). The yield product would be shorter, with 1001-bp length, as it would not include the insertion cartridge, and therefore, equivalent to the vector virus gene region. On the other hand, an opposite situation could also occur, in which a 288-nucleotide alignment takes place in the encoding region of the stem and anchor domains of E-protein with the negative-strip supplementary homology area of the heterologous cartridge expression. Accordingly, it would be produced a longer PCR fragment, with 3059 bp, including the replicate EGFP gene (Figure 16D), which, by its turn, is also detected (Figure 16 A), although to a lesser extent because of its less effective synthesis due to its longer length. Because of the manner in which this alignment occurs and these fragment syntheses, they produce other minor products, as can be evidenced in Figure 16, bands 4 e 6.

Such initial evidences forced such samples analyses by other supporting method to assess the viral genetic stability, since the sole use of RT-PCR method would be insufficient to its confirmation. Thus, the respective

5   samples to different serial passages were analyzed by flow cytometry approach, which would enable the concurrent viral antigen and EGFP detection. A direct signal relation between them, using as a common denominator the quantity of infected cells, would indicate the presence and

10 functionality of heterologous cartridge expression cartridge. Monolayers with approximately 10 Vero Cells were infected with control and recombinant virus. After 72 hours of viral infection (in a 0.02 medium), these monolayers were twice washed with 1 mL of PBS/ 1 mM EDTA, and removed

15 by cellular trypsination and submitted to 2.000 g centrifugation for 7 minutes at 4°C. The cells were then resuspended in a 2% paraphormaldehyde solution, and incubated for 20 minutes at 4 °C. It was added 0.5 mL of a PBS/ 1 mg/mL BSA solution, containing 15% saponine, and the

20 cells were centrifuged at 2.000 g for 7 minutes at 4 °C. It was then added 1 mL of PBS/BSA/15% saponine solution, and the cells incubated for 10 minutes at 4 °C and centrifuged at 2.000 g for 7 minutes. This cell suspension was treated with 20 µL of anti-yellow fever antibody (yellow fever 17D

25 hyperimmune ascitic fluid – mouse – NIAID, code number V525701-562) diluted in a 1:80 ratio in a PBS/BSA/15% saponine solution for 1 hour at 4 °C. It was then added 1 mL of PBS/BSA/15% saponine, and after a 2.000 g centrifugation was performed for 7 minutes and the cells

incubated with 20 µL of anti-mouse antibody conjugated with phycoeritrine (DAKO; in a 1:40 dilution in a PBS/BSA/15% saponine solution) for 30 minutes at 4 °C. After adding 1 mL of a PBS/BSA/15% saponine solution, the cells were

5   centrifuged at 2.000 g for 7 minutes, and the supernatant discarded, and after the cells were submitted to a suspension in 0.3 mL of a 2% paraphormaldehyde solution. In order to obtain data, these cells were centrifuged at 2000 g for 7 minutes and suspended in 0.3mL of a PBS solution

10   and an analysis was made with the FACScalibur flux cytometer (Becton and Dickinson, USA). The data produced by the cytometer were assessed using the FlowJo Software (TreeStar Inc, USA).

Continuous seeding of this virus in Vero cell

15   monolayers was performed to assess 17D/Esa/5.1 glic virus genetic stability. In Panel A of Figure 17, it is shown a schematic figure of viral regeneration and subsequent passages (10) of the virus which was obtained after the transfection and in Panel B, the Vero cells percentage

20   which presented fluorescence to vaccine antigen and EGFP after the infection by recombinant 17D/Esa/5.1 glic virus or only by the vector virus. These results consist of two independent series of serial passages; 5P1 and 10P1 samples corresponding to one of the series and 5P2 and 10P2,

25   corresponding to the other independent experiment.

Based on flow cytometry analysis (Figure 17B), it was calculated a percentage ratio to each sample, that is the relationship among infected cells which show double marking, EGFP's and viral antigens', but only those which

had shown viral antigens without any fluorescence signs within EGFP detection range. This figure represents therefore the percentage of infected cells by 17D/Esa/5.1 glic FA virus expressing EGFP protein. It was also

5   performed an electrophoresis analysis in 0.8% agarose gel of the obtained fragments by RT-PCR reaction to identify these samples, using the initial elements (RG174 - SEQ ID 16 e RG19 - SEQ ID 17) from viral RNA (Figure 17C). Virus related to passages shown in Panel A, and present in the

10 supernatant of used cultures to obtain cytometry data (Panel B) were used to extract RNA. Band 1, and regenerated E200 vector virus from pT3 plasmid. Band 2-7, RT-PCR products maximized from 17D/Esa/5.1 glic virus RNA in different passage levels. Bands 2 and 3, RT-PCR from RNAs

15 of viral stock solutions obtained from one (1P) or two passages (2P) of the resulting transfection virus, respectively. Bands 4 - 5 e 6-7 represent RT-PCR products, which were obtained from virus RNA in the fifth and tenth passages ·of two independent strains (5P1 and 5P2; 10P1 and

20 10P2, respectively).

The concurrent analysis of viral samples, by RT-PCR and flow cytometry methods, was performed to serial passages 1P, 2P, of samples of two independent series of serial passages - (5P1 and 10P1; 5P2 and 10P2), as can be

25 seen in Figure 17.

Flow cytometry analysis revealed that the percentage for positive cells to viral antigens and EGFP, after 17D/Esa/5.1glic virus infection, ranged from 76% to 92 % (Figure 17B, sample 2P and 1P, respectively). This includes

the passages, with 85% of doubled marked cells compared to the average value. In baseline, 17D/E200T3 FA control virus shown 0.8% of double marker cells (Figure 17B). These results suggest that in cells, positive in relation

5   to viral antigen, EGFP is also present. This conclusion is supported by RT-PCR product analysis, using RG 174 (SEQ ID 16) and RG19 (SEQ ID 17) oligonucleotides, due to the presence of 2.0 kb band, indicating that heterologous cartridge expression existed in Vero cell monolayers

10   infected by FA 17D/Esa/5.1 glic virus as far as the tenth consecutive passage (Figure 17C). 1 kb band, evidenced in all RT-PCR reactions using RNA from 17D/Esa/5.1glic virus detected over the passages, may be related to the device described in Figure 16. To confirm this interpretation,

15   Figure 18 shows the same kind of study using a cloned viral population. The transfection supernatant of 17D/Esa/5.1glic 1P virus was placed in Vero cells monolayers plates, with 0,5% agarose coating in 199 Earle's medium enriched by 5% fetal bovine solution (second cellular passage or 2P).

20   After 4-day incubation at 37 °C, it was applied to this coating E 199 Earle's medium containing 0.1% neutral red, in order to allow viewing lyse plates and its isolation by puncturing their coating with a Pasteur pipette, to free the material in sterile PBS and the placement in the plates

25   of approximately 100 µL of this suspension in 24-microwell plates containing approximately 100,000 cells per microwell. This coating would mean a third cell passage, but each lyse plate corresponds to a clone of the original population of 17D/Esa/5.1glic virus.

One of these clones was randomly selected to be submitted to genetic stability analysis and it was named clone 6 (Figure 18). Viral samples of this clone 6 was obtained as far as the fifth tenth continuous passage

5   through Vero cell monolayers and analyzed using RT-PCR and FACS methods (Figure 18), except the first clone sample 3P, which was only analyzed by RT-PCR, due to the little amount of sample that was obtained (Figure 18 C). In Figure 17, Panels A to E, it is shown electrophoresis profiles of RNA

10   amplification by RT-PCR and fluorescence distribution of 17D/Esa/5.1 glic virus, achieved by the transfection in passage 1 (Panel A) and passage 2 (Panel B), and passages 5 and that of clone 6 (Panels D and E, respectively). In Panel C, it is shown the electrophoresis profile of RNA

15   amplification by RT-PCR in clone 6 original stock solution of 17D/Esa/5.1 glic virus, which was used for serial passages shown in Panels D and E, and in Panel F, Vero cell percentage showing fluorescence to vaccine antigens and EGFP after infection by 17D/Esa/5.1 glic virus from passage

20   1 (1P) or two (2P) of the resulting transfection virus, and clone 6 in passages 5 (5P) and 15 (15P).

In all analyzed samples, it was possible to detect the same band pattern previously established, that is, the occurrence of 2.0 kb and 1.0 kb bands, even in recently

25   cloned viral preparation 3P (Figure 18C), confirming this RT-PRC technique limitations to assess genetic stability of heterologous insertion in the genome of 17D/Esa/5.1glic virus. On the other hand, flow cytometry analysis of Vero cells, infected by these different viral samples, indicates

once again the insertion stability of EGFP gene, since 95%

of the infected Vero cells by the viral preparations

corresponding to passages 5 and 15 of clone 6, expressed

viral antigens and EGFP (Figure 18E).

5   Example 10. Cartridge expression of heterologous expression

for chimeric flavivirus

**Creation and characterization of chimeric virus prM-E
17D/D4.**

       We constructed the chimeric virus 17D/DEN4/FA using

10   prM/E genes of dengue 4 virus, named Venezuela 88. DEN4

Ven88 virus was isolated from blood sample of a patient who

had classical dengue disease, by direct spreading in C6/36

cells. The virus sample, as well as the prM/E gene sequence

of this virus, were gracefully provided by Dr. F. Liprandi

15   (IVIC, Venezuela). The viral chimeric was constructed

using 2-plasmid system of FA infectious clone (Rice, C. M.,

A. Grakoui, R. Galler, and T. J. Chambers. 1989.

Transcription of infectious yellow fever RNA from full-

length cDNA templates produced by in vitro ligation. New

20   Biol 1:285-96).

       The prM/E genes of dengue 4 virus were amplified from

extracted   RNA   of   infected   cells   with   partially

supplementary synthetic oligonucleotides to edge 5' of prM

gene   of   Den   4   virus   (RG   295:   5'-

25   GCTTGATTCCC<u>ACCGGT</u>ATGGCGTTTTCCCTCAGCACAAGAGATGGC 3'; SEQ ID

No. 18) and to region 5' of gene E (RG 296: 5'

GGGCAGA<u>ATGCAT</u>GGCTCC 3'; SEQ ID No. 19), which code *Age*I and

*Nsi*I sites, respectively. This fragment was cloned in pG1/2

plasmid (Galler, R. and Freire, M.S. 2003. Vaccines against

infections caused by YF virus; infectious cDNA, method for producing a recombinant YF virus from the YF infectious cDNA and plasmids to assemble the infectious cDNA. US Patent 6,589,522) to create pG1/2 DEN4 plasmid. The

5 assembly between gene C from FA and dengue prM was conducted at the cleavage level by signalase (Caufour, P. S., M. C. Motta, A. M. Yamamura, S. Vazquez, Ferreira, II, A. V. Jabor, M. C. Bonaldo, M. S. Freire, and R. Galler. 2001. Construction, characterization and immunogenicity of

10 recombinant yellow fever 17D-dengue type 2 viruses. Virus Res 79:1-14). The remaining part of dengue 4 gene E was cloned after amplifying it with RG 297 oligonucleotides (5' GGAGCCATGCATTCTGCCC 3', including NsiI site; SEQ ID No. 20) and   RG   298   (5'

15 GACGCCACACAACCCATGTCGGCGCCAACTGTGAAGCCCAGAAACAGAG   3´, including NarI site; SEQ ID No. 21) in pYFMT3 plasmid (Galler, R. and Freire, M.S. 2003. Vaccines against infections caused by YF virus; infectious cDNA, method for producing a recombinant YF virus from the YF infectious

20 cDNA and plasmids to assemble the infectious cDNA. US Patent 6,589,522), which contains a NarI site within E and NS1 proteins, producing pT3D4Ven88 plasmid. The cDNA that contains all 17D/DEN4 genome was constructed from the liasion of three pieces: NotI-NsiI derived from pG1/2DEN4

25 (with SP6 promoter, FA region 5' NTR -C and DEN4 prM-2/3 E), NsiI-MluI, derived from pT3D4Ven88 (encoding region 3' of DEN4 gene E and FA gene NS1), MluI-NotI derived from FA 17D/DEN1 clone (which has the remaining part of the FA genome, cloned in low copy number vector pACNR1180; Mateu,

G.P. R.S. Marchevsky, F. Liprandi, M.C. Bonaldo, E.S.F. Coutinho, M. Dieudonné, E. Caride, A.V. Jabor, M.S. Freire, R. Galler. 2006. Construction and biological properties of Yellow Fever 17D/ Dengue type 1 recombinant virus. Trans R
5 Soc Trop Med Hyg, no prelo; Bonaldo, M. C., R. C. Garratt, P. S. Caufour, M. S. Freire, M. M. Rodrigues, R. S. Nussenzweig, and R. Galler. 2002. Surface expression of an immunodominant malaria protein B cell epitope by yellow fever virus. J Mol Biol 315:873-85). All plasmids were
10 cultivated in *E.coli* XL-1 Blue.

It was obtained several transformers and 10 completed clones were identified after transforming each strain, suggesting the genetic stability of the construction. Four of them were selected as they had the proper physical map,
15 linearized with XhoI, and used to *in vitro* transcription. RNA was used to viral regeneration by electroporation of Vero Cells. At first, viral viability evidences were viewed by cytopathic effect. The 4 identified clones generated 17D/DEN4 virus (clones 1, 2, 4 and 5), 5-7 days after
20 electroporation. RNA was extracted from the monolayers, and used to RT-PCR reactions. Limitation analysis and amplicons nucleotide sequencing confirmed the *chimeric structure of the virus*. It was performed a new passage, from which working stock viral solution were produced (titration
25 around 6.0 $log_{10}PFU/ml$). For further working steps, involving molecular cloning of EGFP protein expression cartridge of the chimeric virus Den4/FA genome, it was selected clone number 5, which was named pNSK Den4/FA plasmid.

Molecular cloning of EGFP protein expression cartridge in chimeric virus prM-E 17D/D4 genome

Approximately 10 µg of pGEM-T plasmid, obtained as described in example 2 of this document, containing the
5   expression cartridge of EGFP protein, which was digested with 3U of Nar I (Promega). This sample concentration was increased by ethanol-precipitation and resuspended in electrophoresis sample buffer, in addition to being submitted to 1% agarose gel electrophoresis. DNA band
10   containing 1029 bp (SEQ ID No. 4) was purified from the gel by DNA purification system of agarose gels (Qiagen). The material was quantitatively assessed by spectrophotometry at 260 nm, and analyzed by 1% agarose gel electrophoresis.

A DNA fragment of approximately 1 kb, including Nar I
15   cohesive edges, was linked to pNSK Den4/FA plasmid, previously linearized with restriction enzyme Nar I. As previously described, this site is situated in this plasmid exactly in the linking point within encoding genes to E protein of dengue 4 viruses and NS1 of yellow fever virus.
20   The liking was made with pNSK Den4/FA plasmid, digested with Nar I, in 20-fold molar excess of insertion containing EGFP gene and the gene of the enzyme T4 DNA liase (Invitrogen). The equivalent amount of 10 ng of liason was transformed into *E.coli* DH5α (Stratagene), which was
25   transferred to plaques with LB 1.5 agar selective medium, containing 25 µg/mL of ampicilin. It was made mini DNA plasmid preparations, from bacteria colonies :resistant to ampicilin; and these DNA plasmid preparation were submitted to Nar I digestion to confirm the cartridge cloning. The

correct direction of the insertion was verified by the nucleotide sequencing, using synthetic RG 19 oligonucleotide (SEQ ID No. 17). Thus, it was obtained a recombinant pNSK Den4/FA/Esa/EGFP plasmid, with 14.498 base

5   paired-length, as illustrated in the map shown in Figure 19, and detailed in SEQ ID 22 sequence, in which viral genomic cDNA is included within the 639 and 12,543 nucleotide positions, corresponding to a 11,905 nucleotide viral genome, according to SEQ ID 23. The positions inside

10   the genome of 17D/FA/Den4/Esa/EGFP/6 virus of the sequences of C, prM and E genes and the sequence constituents of the EGFP protein expression cartridge – The 27 encoding nucleotides of NS1 protein N-terminal, the EGFP gene and the 288 nucleotides in the stem anchor part – are shown in

15   Figure 20B. It should be noted that the heterologous insertion is allowed by Nar I sites used in molecular cloning of flavivirus genome, as well as by two stem-anchor regions: the first one located in the 5´ part of EGFP gene, is referred to the stem anchor part constituent of the

20   encoding gene for E protein of dengue 4 dengue, and the second one, to the stem anchor part constituent of the encoding gene for E protein of yellow fever virus, part of the heterologous cartridge expression (Figure 20A).

Obtaining chimeric virus 17D/Den4/FA/Esa/EGFP

25   The pNSK Den4/FA/Esa/EGFP plasmid was digested by the enzyme Xho I, according to the manufacturer specifications (Promega) and the resulting cDNA mould preparation was precipitated with ethanol, and resuspended in Tris-EDTA buffer, pH 7.5, without nucleases. The preparation sample

was submitted to agarose gel electrophoresis to detect its
mould and quantification. The equivalent amount to 100 ng
of linearized mould was used to an *in vitro* transcription
step of the viral RNA, using the enzyme SP6 RNA polymerase

5   (Ampliscribe, Epicentre Technologies), according to
protocols previously established (Galler, R. e Freire, M.S.
2003. Vaccines against infections caused by YF virus;
infectious cDNA, method for producing a recombinant YF
virus from the YF infectious cDNA and plasmids to assemble

10 the infectious cDNA. US Patent US 6,589,522). The integrity
of the RNA transcripts was verified, using 0,8%/TAE agarose
gel electrophoresis. Viral RNA was transfected into Vero
cell monolayers, in the presence of Lipofectamine
(Invitrogen), which has a concentration of 20 µg/mL in PBS.

15 The culture supernatant was collected after establishing
cytopathic effect, and used to obtain viral stock
solutions.

**Kinetics assessment of virus growth of 17D/Den4/FA/Esa/EGFP
virus using Vero cell monolayers.**

20   The growth capacity of the obtained recombinant
17D/Den4/FA/Esa/EGFP virus was analyzed, in relation to
vaccine FA17DD virus and parent chimeric 17D/Den4/FA virus,
by means of infection in Vero cell monolayers. Three
independent experiment were performed in respect of the

25 viral spreading kinetics in Vero cell monolayers (62,500
cells/cm^2), in an infection multiplicity (m.o.i) of 0.02.
Aliquots of cellular supernatant at 24, 48, 72, 96, 120 and
144 hour post-infection (p.i.) were sampled and tittered
(Figure 21).

The viral growth peaks of FA 17DD and 17D/Den4/FA occur 72 hours after infection, at 7.17 and 6.69 log10 PFU/mL, respectively. When these two viruses kinetics profiles are compared to that of recombinant

5 17D/Den4/FA/Esa/EGFP virus, it can be concluded that the later has a less marked growth, with viral production of 6,31 log10 PFU/mL 96 hours after infection (Figure 21).

Genetic stability of 17D/Den4/FA/Esa/EGFP virus by serial passages in Vero cell monolayers.

10 The genetic stability of the chimeric 17D/Den4/FA/Esa/EGFP virus insertion was assessed by two series of independent passages in Vero cell monolayers. After *in vitro* transfection of synthesized viral RNA and the occurrence of cytopathic effect, viral supernatant was collected and the

15 obtained viral particle preparation named first cellular passage or 1P, and it was then used to a further infection of Vero cell monolayers in a 62,500 cells/cm^2 density. The second cycle infection of this viral supernatant was named second cellular monolayer passage or 2P, and it was then

20 collect, measured and stored at -70°C, after the occurrence of the cytopathic effect, approximately 96 hours after the infection. Then, it was performed the titration of this suspension in order to proceed to the next serial infection in a 0.02 moi. Afterwards, it was established two series of

25 consecutive viral infection in Vero cell monolayers, named P1 and P2. This procedure was continuously repeated until the twentieth serial passage was reached.

Passage samples 1P, 2P, 5P1, 5P2, 10P1, 10P2, 15P1, 15P2, 20P1 and 20P2 were submitted to viral RNA extraction

by Trizol LS method (Invitrogen), and then the RT-PRC
procedure, using enzyme M-MLV (Promega Corporation), was
performed to achieve the syntheses of simple strip cDNA and
Tag polymerase enzyme to allow the PCR reaction (Promega

5   Corporation), according the manufacturer specifications,
aiming to verify the heterologous insertion integrity.

It was used RG 367 (SEQ ID 24) oligonucleotides,
positive and corresponding direction to 1594-1612 genomic
region of dengue 4 virus and RG 19 (SEQ ID 17)

10   oligonucleotides, negative and corresponding direction to
2619 a 2639 genomic region of yellow fever virus. In the
genome of 17D/Den4/FA/Esa/EGFP virus, these
oligonucleotides correspond to 2276-2294 and 4301-4321
genomic regions, respectively. The intended length of DNA

15   fragment, containing EGFP heterologous cartridge expression
cartridge would be 2046 base pairs (bp), while this same
region in parent 17D/Den4/FA virus, that is, without EGFP
insertion, would have a 1017 bp-length. As can be noticed
in Figure 22, the band which contains the heterologous

20   insertion is kept as far as the twentieth passage of two
series of independent spreading, indicating the
construction stability expressed by the recombinant
flavivirus. Minimum quantities of 1,017 bp band can be
noticed, reflecting the spurious amplification detailed in

25   example 9.

EXAMPLE 11: Heterologous protein expression fusioned to
genomic region corresponding to partial stem and anchor
domains of E protein

Heterologous nucleotide sequences can also be cloned and expressed in yellow fever vector virus, in a manner that its 5' portion keeps nucleotides in the 5' portion of its NS1 gene or of others virus and sequences of equivalent

5   function, and in its 3' portion, the genomic region correspondent to stem and anchor domain parts of E protein of this vector virus. Thus, a yellow fever 17D virus was obtained, in which it was cloned the gene that encodes the reporting EGFP protein (SEQ ID 2) among encoding genes to E

10   and NS1 proteins, in such a manner that in its 5' encoding edge, 27 corresponding nucleotides to NS1 protein N-terminal (SEQ ID No. 1) were fusioned, and to its 3' edge, the genic region of 1988 nucleotides (SEQ ID No. 25), corresponding to partial stem domain, only H2 region,

15   followed by anchor region, containing the two transmembrane region, totalizing 66 amino acids (SEQ ID No. 26), having as a result a 939 bp-heterologous gene (SEQ ID No. 29), which corresponds to a protein with 313 amino acids (SEQ ID No.30). The precursor polyprotein resulting from this

20   recombinant FA virus would be properly cleaved in the regions which side the heterologous protein, because of sign sequences presence expressed in E protein and heterologous protein C-terminal, in an analogous manner as described in example 2.

25   **Synthesis and cloning of EGFP expression cartridge**

In order to obtain an expression cartridge for EGFP protein, it was firstly synthesized, using PCR, two DNA fragments:

(1) a 784 bp-DNA fragment, containing EGFP gene, using
the pEGFP-C2 plasmid (BD Biosciences Clontech) and the
synthetic RG 328 (SEQ ID No. 9) and RG 332 (SEQ ID No. 27)
oligonucleotides. The RG 328 (SEQ ID No. 9), of positive

5   polarity, contained, in sequence to 15 nucleotide-genic
regions corresponding to E protein carboxyterminal, 27
nucleotides corresponding to the first nine amino acids of
NS1 protein; besides 20 nucleotides of EGFP 5´edge. The RG
332 (SEQ ID No. 27), of negative polarity, contains, in

10 sequence to 22 nucleotide-genic regions of EGFP gene 3'
edge, 28 nucleotides corresponding to H2 region N-terminal
of the stem and anchor domains of E protein.

      (2) A second fragment with 247 bp was obtained, using
T3 plasmid and a synthetic RG 33 oligonucleotides, positive

15 polarity (SEQ ID No. 28) with 50 nucleotides corresponding
to a region with 22 encoding nucleotides of EGFP protein C-
terminal and 28 nucleotides, corresponding to H2 N-terminal
region of the stem domain and RG 331 (SEQ ID No. 12),
inverted direction, corresponding to 19 nucleotides which

20 encode the carboxy terminal of TM2 followed by 27
nucleotides encoding the NS1 protein N-terminal. The
resulting DNA fragment consists of, direction 5´ to 3´ of
the encoding strip, 22 nucleotides, corresponding to the
carboxy terminal of EGFP protein, followed by 198

25 nucleotide genic region (SEQ ID No. 25), which encodes 66
residual amino acids (SEQ ID No. 26), corresponding to
truncated stem domains (only H2 region) and E protein
anchor domain (2255 to 2452 FA genomic position); finally,
followed by the genic region with 27 nucleotides,

corresponding to 9 residual amine-terminal of NS1 protein
(2453 to 2479 FA genomic position).

The fusion of these two DNA fragments, to generate
EGFP protein expression cartridge to be cloned in the
5   genome of the yellow fever virus, was carried out by PCR
reaction with equivalent molar amounts of fragments with
784 bp and 247 bp, in the presence of 20 µM RG 328 (SEQ ID
No. 9) and of RG 331 (SEQ ID No. 12). All PCR reaction was
performed with the enzyme Platinum *Pfx* Polymerase
10 (Invitrogen), pursuant to the manufacturer recommendations.
The reaction products were analyzed in 1% agarose gel
electrophoresis and later purified by PCR product purifying
system (Qiagen).

The resulting fragment with 939 pb was cloned in pGEM-
15 T plasmid (Promega), as specified by the manufacturer. *E.
coli* MC1061 competent bacteria were transformed with 10 ng
of liaison and placed on selective medium plates (1.5% Agar
LB with 50 µg/mL of ampicilin). Plasmid DNA preparations of
these bacterial clones were obtained and submitted to
20 digestion by the enzyme Nar I, in order to confirm the
cartridge cloning of 939 bp-DNA (SEQ ID No. 29) that
encodes a protein with 313 residual amino acids (SEQ ID No.
30). One of these bacterial clones was selected, and its
plasmid DNA was sequenced to confirm the direction and
25 integrity of its insertion.

Approximately 10 µg of pGEM-T plasmid, with expression
cartridge of EGFP protein, was digested by 3U of Nar I
(Promega). The sample was concentrated with ethanol-
precipitation, and resuspended in electrophoresis sample

buffer, in addition to being submitted to 1% agarose gel
electrophoresis. DNA strip with 939 bp (SEQ ID No. 29) was
separated from the gel using the DNA purifying system with
agarose gels (Qiagen). The material was quantified by

5   spectrophometry at 260 nm, and analyzed by 1% agarose gel
electrophoresis.

The DNA fragment with approximately 1 kb, containing
Nar I cohesive edges I, was linked to T3 vector plasmid,
which includes partial cloned viral cDNA (1373 to 9428

10 genomic position), previously digested by Nar I, in a
medium with 20-fold molar in excess of the insertion
containing EGFP and enzyme T4 DNA liaise genes
(Invitrogen). The corresponding amount to 10 ng of liaison
was transformed into *E.coli* Sure (Stratagene), which was

15 placed in plaques in a 1.5% Agar LB selective medium, with
50 µg/mL of ampicilin. It was then prepared mini plasmid
DNA preparations from bacteria colonies resistant to
ampicilin; and plasmid DNA preparations which had a higher
length than the original pT3 control were submitted to Nar

20 I digestion to confirm the cartridge cloning. In order to
verify the proper direction of the insertion nucleotide
sequencing was performed. Accordingly, recombinant pT3
Esa$_{trun}$ EGFP plasmid was obtained. In Figure 23, it is shown
the physical map of recombinant T3 Esa$_{trun}$ EGFP plasmid.

25 **Mould preparation of viral cDNA viral**

cDNA mould, used to obtain recombinant FA 17D virus,
was achieved using the same methodology as described in
example 3 of this document. Accordingly, pT3/Esa$_{trun}$/EGFP
and pE200$_{glic}$ plasmids were cleaved with restriction enzymes

Nsi I and Sal I (Promega), according to conditions as
recommended by the manufacturer. Approximately 10 μg of
each plasmid were digested with both enzymes. The cleavage
was monitored by analysis of aliquots equivalent to 200 ng
5 of DNA in 0.8% agarose gel electrophoresis in a 0,8% TAE
buffer. After complete cleavage, the enzymes were
inactivated by heat treatment. The cleavage products
NsiI/SalI of these plasmids were linked by T4 DNA liaise
(Epicentre Technologies), according to conditions
10 established by the manufacturer. The linearization of cDNA
different moulds was made using restriction endonuclease
Xho I, under condition as established by the manufacturer
(Promega). The resulting products were subjected to ethanol
precipitation and resuspended in a Tris-EDTA buffer
15 solution with pH 7.5 without nucleases. A sample of each
preparation was analyzed by agarose gel electrophoresis to
detect its mould and quantification. The preparations were
stored at -20°C until an *in vitro* transcription step.
Obtaining FA virus from viral cDNA: transcription and
20 **transfection steps**

     Using cDNA moulds, which represent the complete
genome, including plasmid sequences $pE200_{glic}$ and
$pT3/Esa_{trun}/EGFP$, viral RNA preparations were obtained by *in
vitro* transcription system of RNA SP6 (AmpliScribe SP6;
25 Epicentre Technologies). The *in vitro* synthetized RNA
preparations were analyzed by 0.8% agarose gel
electrophoresis in a TAE solution. Aliquots of these RNA
preparations were transfected with Lipofectamine
(Invitrogen Life Sciences) in Vero cell monolayers, as

described by Bonaldo and contributors (Bonaldo, M. C., R.
C. Garratt, P. S. Caufour, M. S. Freire, M. M. Rodrigues,
R. S. Nussenzweig, and R. Galler. 2002. Surface expression
of an immunodominant malaria protein B cell epitope by
5 yellow fever virus. J Mol Biol 315:873-85).

RNA Transfection synthesized *in vitro*

The transfection step was performed in a similar
manner as described in the US Patent 6,171,854 document.
The viral RNA transfection synthesized *in vitro* originates
10 a recombinant virus, with the capacity to grow in Vero
cells. This new recombinant yellow fever virus was named
$17D/Esa_{trun}/4_{glic}$. Its detection was achieved when cytopathic
effect appeared in the cellular monolayer in phase contrast
microscopy. The detection of EGFP protein expression by
15 this virus was performed within a time range of 24, 48, 72,
96 and 120 hours in Vero cells monolayers infected by
$17D/Esa_{trun}/4_{glic}$ virus with a 0.1 m.o.i using fluorescence
microscopy at 488 nm.

The cellular monolayers were washed twice with PBS,
20 and fixed with 4% paraphormaldehyde solution with 0.1 M
dibase phosphate buffer for 10 minutes, and washed once
again with 0.2 M dibase phosphate buffer. After fixing
them, they were assembled in plates and seen using a Nikon
microscope (E600 eclipse model). The highest fluorescence
25 detection of EGFP protein expressed by $17D/Esa_{trun}/4_{glic}$
virus was at 72 and 96 hours after infection, similarly to
$17D/Esa/5.1_{glic}$ virus, which has its stem anchor region
completely fusioned to this heterologous protein
carboxyterminal (Figure 24).

Figure 25 shows, in a schematic manner, the viral genome region, included within prM protein and NS1 protein encoding genes in the recombinant 17D/Esa$_{trun}$/4$_{glic}$ virus, detailing amino acid sequences of the truncated stem anchor

5   region associated to the heterologous expression cartridge, as well as restriction enzyme Nar I sites which side this region, and were used in the molecular cloning of this cartridge in the infectious clone of FA 17D virus. The location of prM, E genes, of heterologous cartridge in the

10   genome of recombinant 17D/Esa$_{trun}$/4$_{glic}$ virus - with their respective domains (27 nucleotides of NS1 gene, EGFP gene and truncated stem and anchor)- and NS1 gene is also shown in Figure 25.

Characteristics of viral spreading: kinetics assessment of

15   **viral growth in Vero cell monolayers**

The capacity to grow of recombinant FA 17D/Esa$_{trun}$/4$_{glic}$ virus was compared to that of recombinant 17D/Esa/5.1$_{glic}$ virus and that of control 17DD viruses- vaccine virus used in human immunization - and experimental vaccine virus

20   17D/E200T3 infecting Vero cell monolayers (62,500 cells/cm^2) in a 0.02 moi. At least three independent experiments were performed for the kinetics of viral spreading under these conditions. Aliquots of cellular supernatant of 24, 48, 72, 96h, 120 and 144 hour post-

25   infection were collected and tittered.

Figure 26 shows graphically the infection kinetics of Vero cell monolayers.

It can be noticed that, while the vaccine FA 17DD virus had a viral growth peak 72 hours post-infection, with

6.88 log10 PFU/mL, not only the experimental vaccine 17D/E200T3 virus, but the recombinant viruses that express EGFP - 17D/Esa$_{trun}$/4glic and 17D/Esa/5.1glic - had very similar kinetics profiles with viral production peaks in 96

5   hours, with values near to 6.40 log10 PFU/mL.

A good spreading in Vero cell monolayers of recombinant 17D/Esa$_{trun}$/4glic and 17D/Esa/5.1$_{glic}$ viruses suggests that the production of recombinant vaccine 17D viruses, to make insertions within E and NS1 proteins in a

10 production level, is feasible.

Although illustrated and described here with reference to certain specific embodiments, the present invention is not meant to be limited only to the details shown. Several modifications can be made on the details within the ambit

15 and reach of equivalents without departing from the spirit of the invention.

CLAIMS

1. Method for production of recombinant virus with coding nucleotides sequences of the whole or part of the heterolog proteins, characterized by the following steps:

5   (a) modification of the heterolog nucleotides sequences in such way they when cloned and expressed in the vector virus, they present in the 5´ region, nucleotides present in the 5´ edge of the gene NS1 of this vector virus or of other virus or equivalent functional sequences, and

10   in its 3´ region, the correspondent genome region in the whole or part of the spheres of the steam and anchor of the protein E of this vector virus or equivalent functional sequences, and not compromising the structure and the replication of the mention vector virus;

15   (b) insertion of the modified heterolog sequences in (a) in the intergene region at the structural protein E level and of the nonstructural NS1 vector virus;

(c) obtention of the non pathogenic recombinant virus and owner of the immunologic properties, having the

20   heterolog sequences integrated in the viral genome according to the insertion described in (b) and, like that, expressing the heterolog antigene in such way that it can induce an appropriate immune response.

2. Method in accordance with the claim 1 characterized

25   by the fact that the virus is wild, attenuated or recombinant.

3. Method in accordance with the claims 1 and 2 characterized by the fact the virus is a Flavivirus.

4. Method in accordance with claim 3 characterized by

the fact that Flavivirus is the virus of Yellow Fever correspondent to 17D strain or its derivatives.

5. Method in accordance with claim 3 characterized by the fact that Flavivirus is the chimeric virus of the Yellow Fever, which has the prM and E of dengue 1,2,3 and 4 virus or of any other chimeric flavivirus obtained by the substitution of genes prM and E.

6. Method in accordance with claim 1 characterized by the fact that the heterolog nucleotide sequences are modified in (a) in such way that when cloned and expressed in the virus, they might have in the 5´ region the nucleotides described in, SEQ ID No. 1 or in its equivalent functional sequences or, ―in the 3´ region, the correspondent genome region to the steam and anchor of protein E as described in SEQ ID No. 3 or its equivalent functional sequences.

7. Method in accordance with claim 1 characterized by the fact that in step (b) the insertion of modified heterolog sequences is performed in the intergenic region E/NS1 of the viral genome.

8. Construct of DNA characterized by being formed essentially by one vector, one genome of genetically stable virus and modified heterolog sequences and introduced in an insertion site in the intergenic region at the level of structural protein E and of the non structural viral NS1 in accordance with any of the claims 1 through 7.

9. A DNA construct in accordance with claim 8 characterized by the fact of having a genetically stable virus genome, correspondent to the genome of virus of

Yellow Fever 17D as mentioned in SEQ ID No. 15 or its equivalent functional sequences, in which the modified heterolog sequences will be inserted.

10. Vector virus of coding nucleotides sequences in the whole or part of heterolog proteins characterized by having these modified sequences in accordance with claim 1 and inserted in the intergene region at the level of structural E protein and of the nonstructural NS1 viral and integrated in the viral genome, because it is not pathogenic, because it has immunologic properties and because it expresses the heterolog antigen in such way that it can induce an appropriate immune response.

11. Virus in accordance with claim 10 characterized for having these mentioned sequences in the intergenic E/NS1 region of the viral genome.

12. Virus in accordance with claim 10 and 11 characterized by the virus is a Flavivirus.

13. Virus in accordance with claim 12 characterized by a Yellow Fever virus correspondent to 17D strain.

14. Virus in accordance with claim 13 characterized by having a genome as presented in SEQ ID No. 13 or its equivalent functional sequences, exempting the nucleotide sequence EGFP, or its equivalent functional sequences, exempting the nucleotide sequence of EGFP protein, which may be substituted by any heterolog sequence.

15. Vaccine composition to immune against Flavivirus and/or other pathogens characterized by consisting essentially of the whole or part of the virus which is obtained in accordance with claims 10 to 14.

16. Vaccine composition to immune against Flavivirus and/or other pathogens characterized by the factor that Flavivirus is the Yellow Fever virus correspondent to 17D strain.

17. Vaccine composition in accordance with claim 15 to 16 characterized by comprising a reasonable amount of virus, and at least one acceptable pharmaceutical carrier.
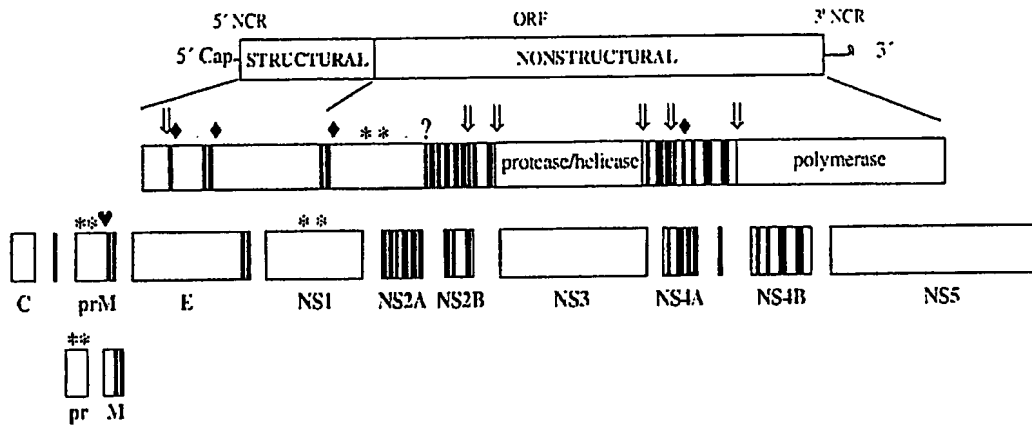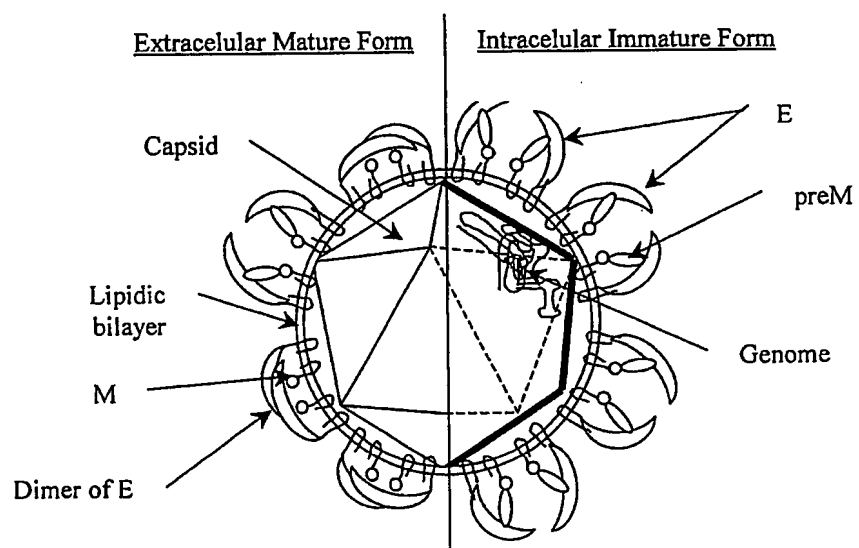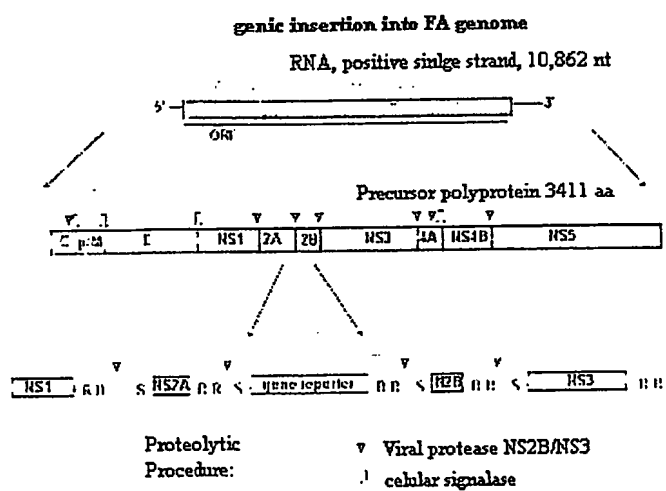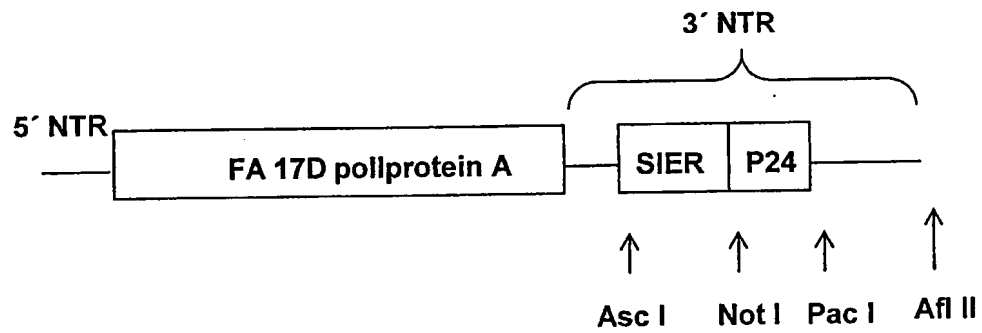
Figure 1

Figure 2

Figure 3

Figure 4

(A)

STRUCTURAL PROTEINS   NON-STRUCTURAL PROTEINS

FURINA

M

(B)
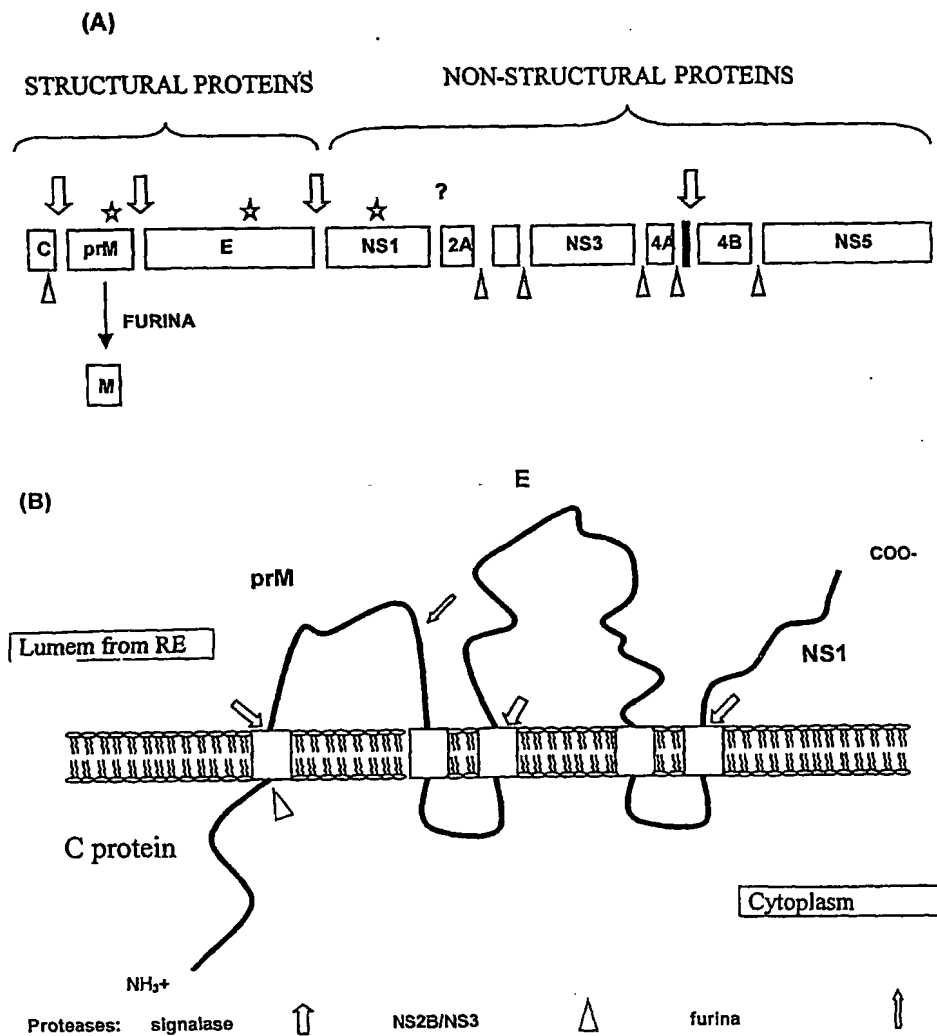
Figure 5

(A)

virus	C-terminal E (7 residues)	N-terminal NS1 (9 residues)
FA	L S L G V G A	D Q G C A I N F G
JE	L A T N V H A	D T G C A I D I T
Den 2	L G V M V Q A	D S G C V V S W K
Den 4	L G F T V Q A	D M G C V A S W S
WN	L S V N V H A	D T G C A I D I G
TBE	M T L G V G A	D V G C A V D T E

(B)

Motif   V X A ↓ D X G C
Consense

Figure 6

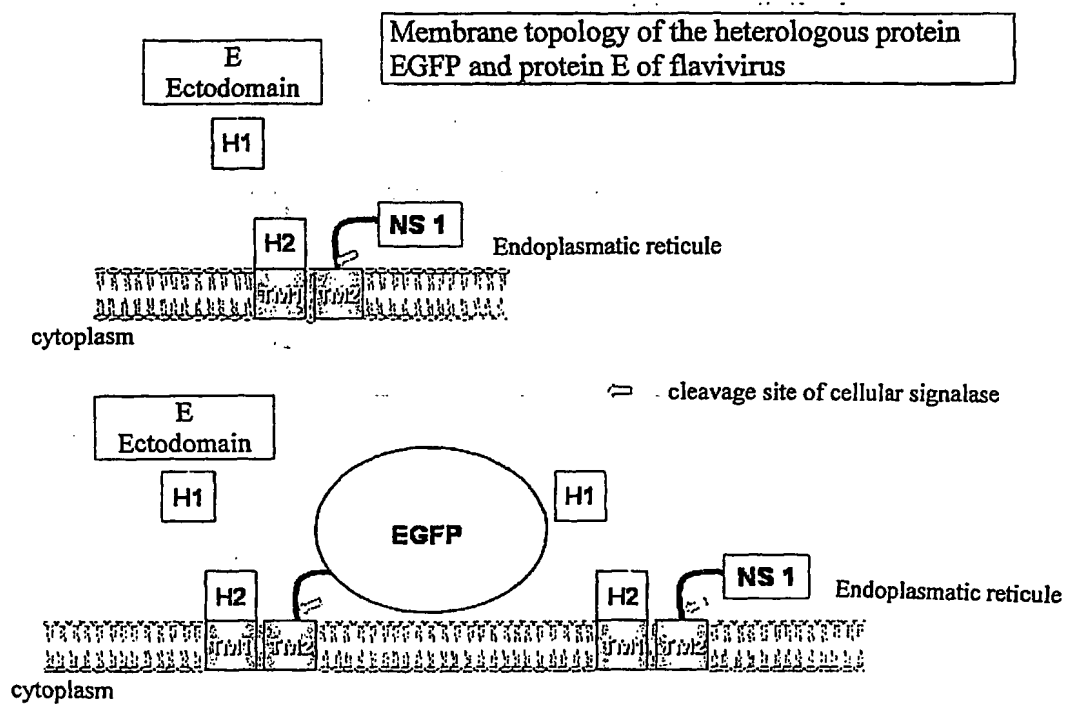Figure 7

(A)

```
                                                                                    96
TBE  RVFQKTKKGIERLTVIGEHAWDFGSAGGFLSSIGKAVHTVLGGAFNSIFGGVGFLPKLLLGVALAWLGLNMRNPTMSMSFLLAGGLVLAMTLGVGA
FA   KLFTQTMKGVERLAVMGDTAWDFSSAGGFFTSVGKGIHTVEGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA
     ::*.:*.**:****:.: ****.*****.*.*.*:::.:..:.:****:.*.:.:.*.*.:*.: ** *****:::*.* .::: ::******

        H1           CS              H2               TMD 1                    TMD 2
```
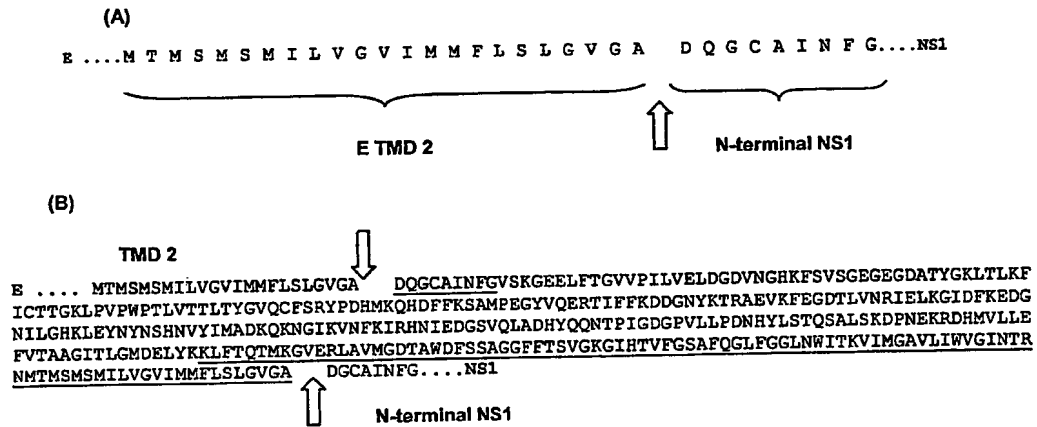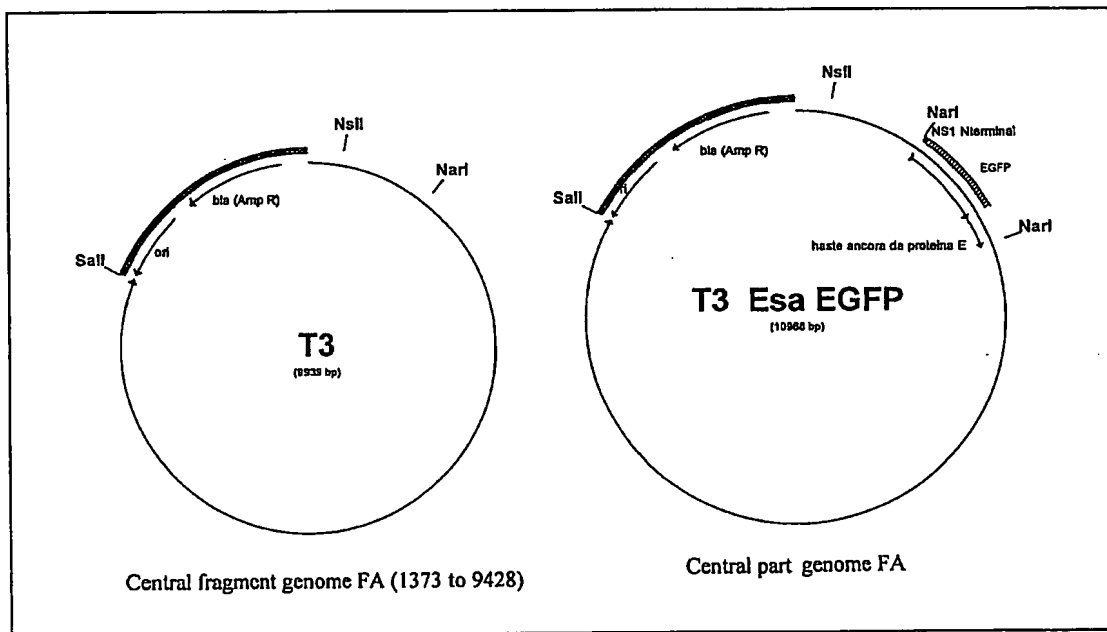
(B)

virus	N-terminal NS1 (9 residues)
FA	D Q G C A I N F G
JE	D T G C A I D I T
Den 2	D S G C V V S W K
Den 4	D M G C V A S W S
WN	D T G C A I D I G
TBE	D V G C A V D T E

Figure 8

Insertion point of protein EGFP into viral
polyprotein

(A)

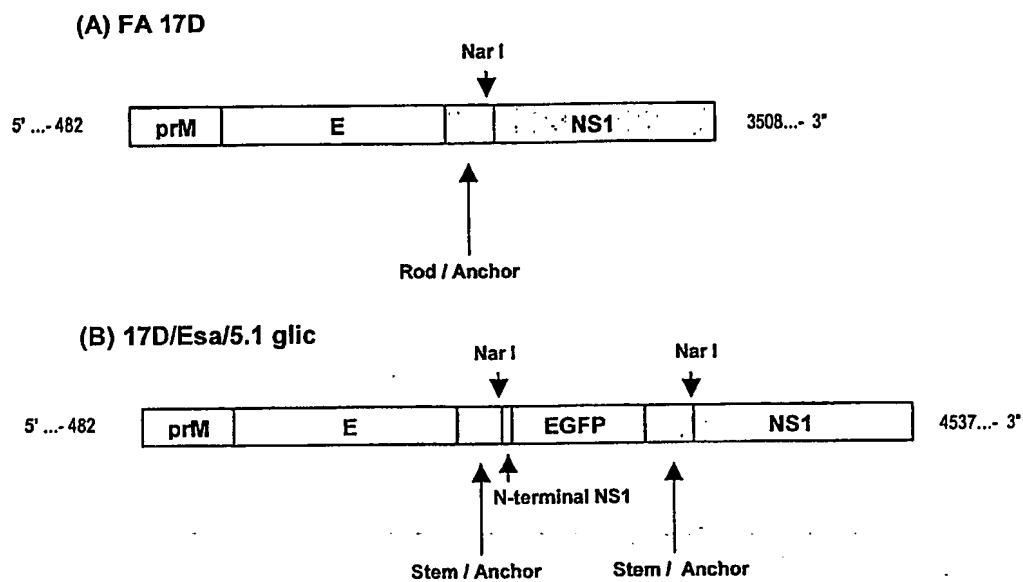E ....M T M S M S M I L V G V I M M F L S L G V G A   D Q G C A I N F G....NS1

E TMD 2   ⇑   N-terminal NS1

(B)

TMD 2   ⇩

E .... MTMSMSMILVGVIMMFLSLGVGA DQGCAINFGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKF
ICTTGKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDG
NILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLE
FVTAAGITLGMDELYKKLFTQTMKGVERLAVMGDTAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTR
NMTMSMSMILVGVIMMFLSLGVGA DGCAINFG....NS1   ⇧

N-terminal NS1

Figure 9

Figure 10

Figure 11

(A) FA 17D

(B) 17D/Esa/5.1 glic

(C)

Gene/ position FA 17D (nt)	5'	3'
PrM	482	973
E	974	2452
E – stem anchor domain	2165	2452
NS1- N-terminal portion	2453	2479
NS1	2453	3508
Gene/ position 17D/Esa/5.1 glic (nt)	5'	3'
PrM	482	973
E	974	2452
E – stem anchor domain	2165	2452
EGFP N- terminal of NS1 portion	2453	2479
EGFP	2480	3193
EGFP – stem anchor domain of E	3194	3481
NS1	3482	4537

Figure 12

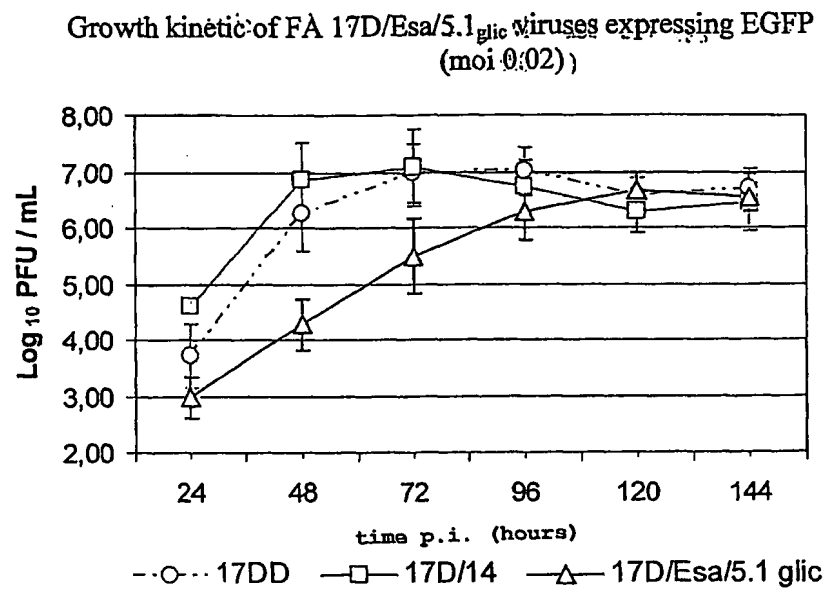Growth kinetic of FA 17D/Esa/5.1 glic viruses expressing EGFP (moi 0.02)

Figure 13

ic Challenge with FA 17DD virus
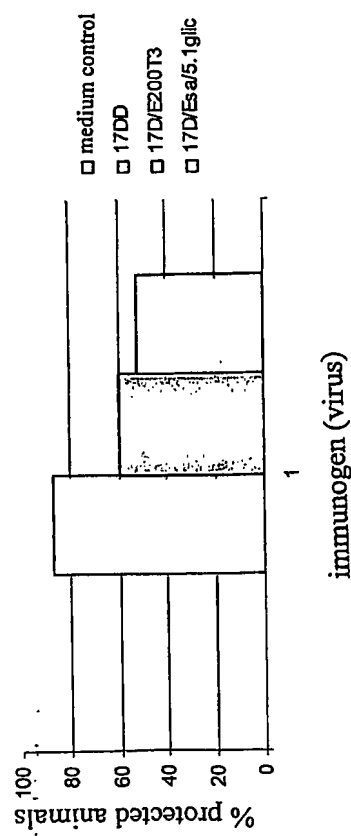
Group	Medium	17DD	17D/E200glicT3	17D/Esa/5.1glic T3
Mortality (%)	100	13,3	40	50
Mean time of overlife ± standard deviation (days)	11 ± 0,94	4 ± 0*	4 ± 0*	4 ± 0*
Number of animals	10	15	15	20
Median dose/immunization	-	62.000	85.000	40.000

Figure 15
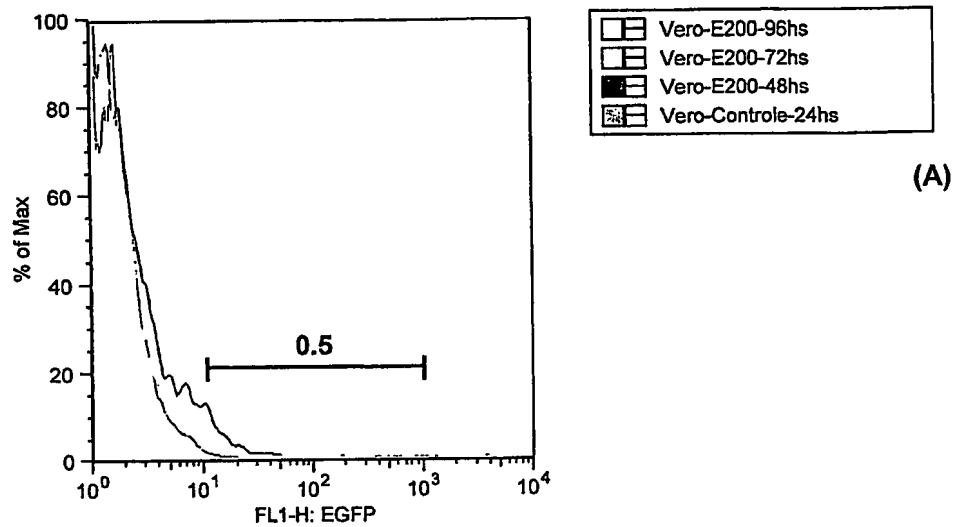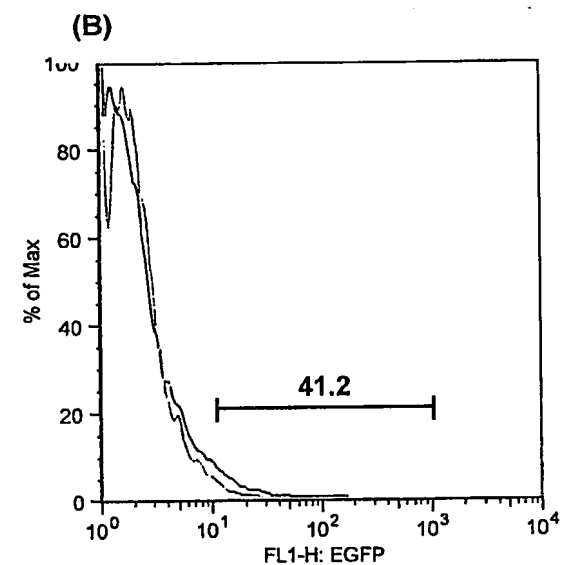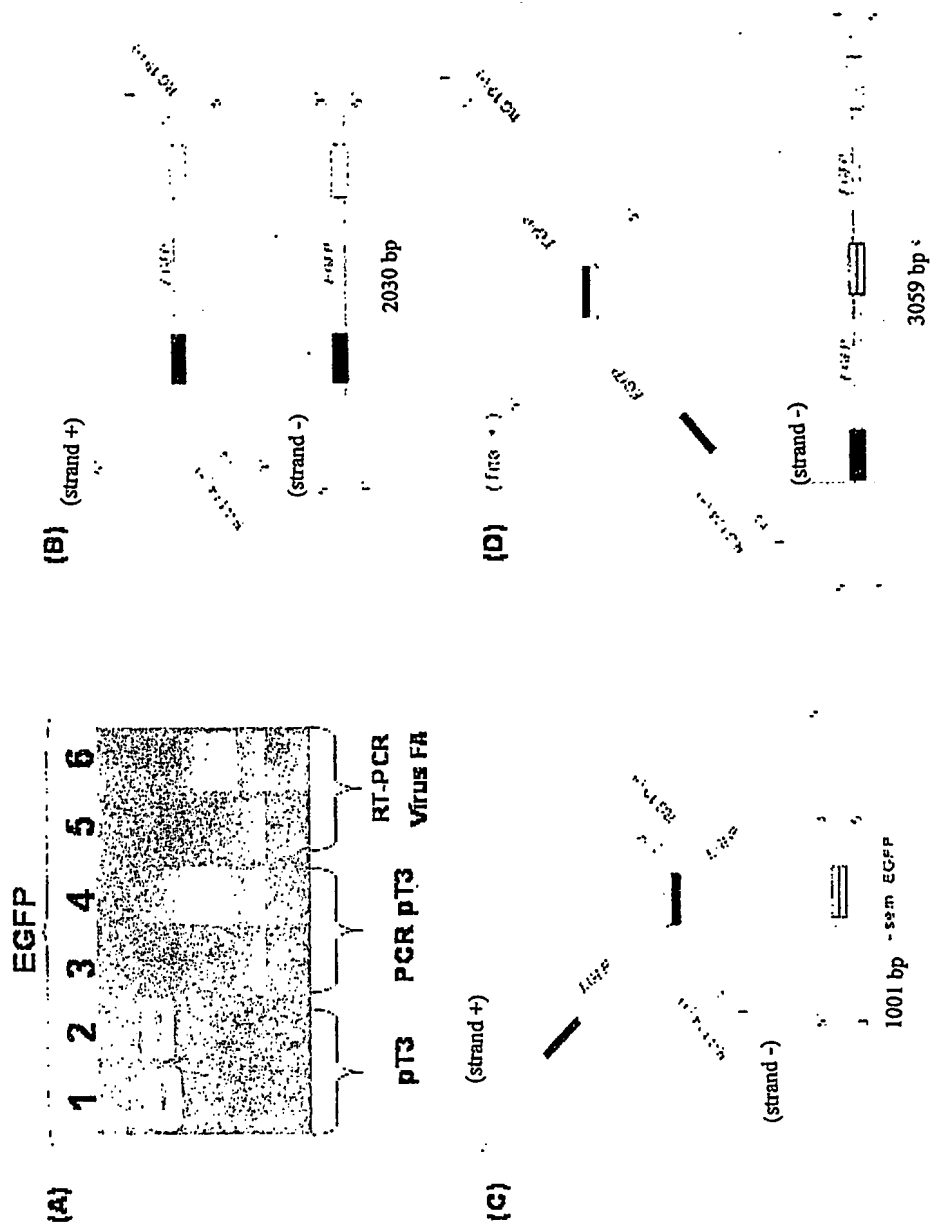
Figure 14

Figure 16

Genetic stability of 17D/Esa/5.1$_{glic}$ after serial passages in monolayers of Vero cells

(B)   Analysis by FACS

(C)

RT-PCR: viral RNA of different samples

2,0 kb

1,0 kb

(A)

Recombinant virus obtained from transfection of the synthesized RNA *in vitro.* 1P

Infection of the cellular monolayer: viral stock 2P

Serial passages of the Viral supernatants until 5th passage 5P

Serial passages of viral supernatants until 10th passage 10P

Passage in Vero cells

RT-PCR and DNA sequencing

Analysis by flux citometry

Figure 17

Figure 18

CDNA genomic Den4-FA
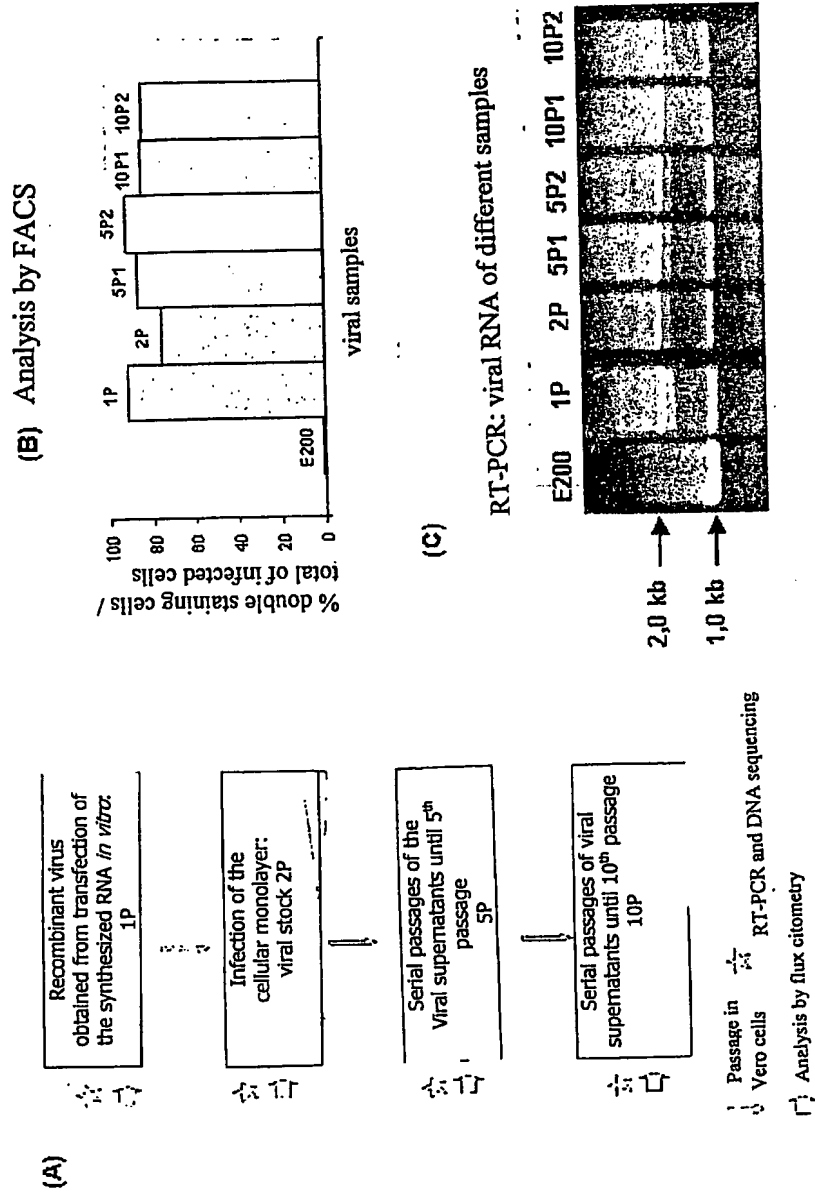
Figure 19

(A) 17D/Den4/Esa/EGFP/6
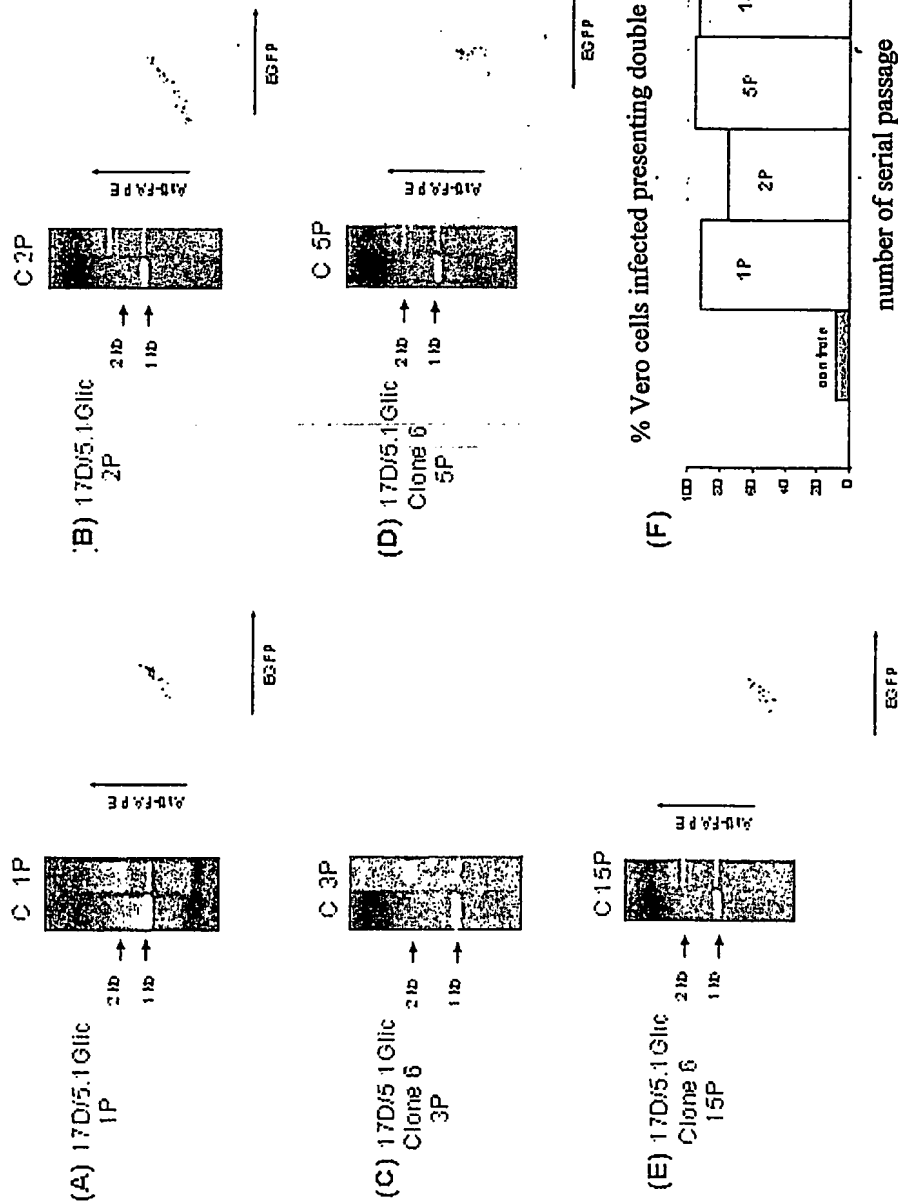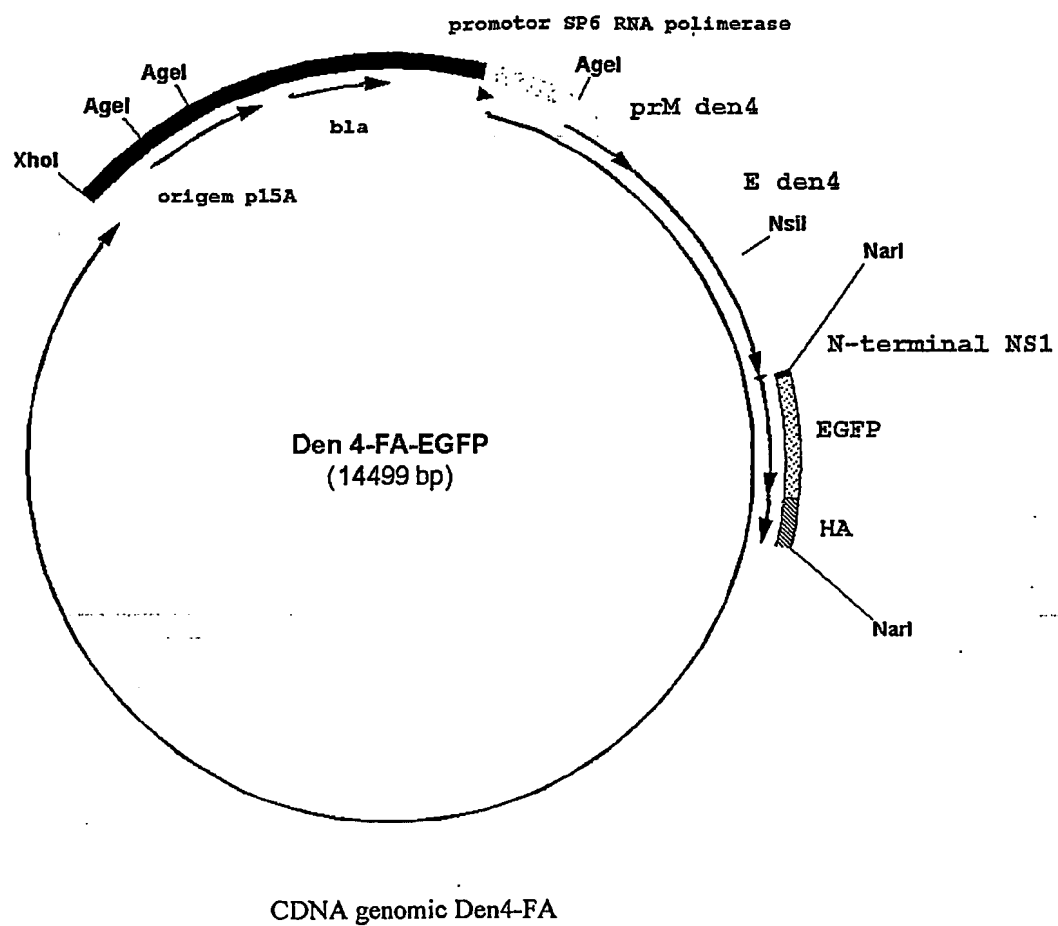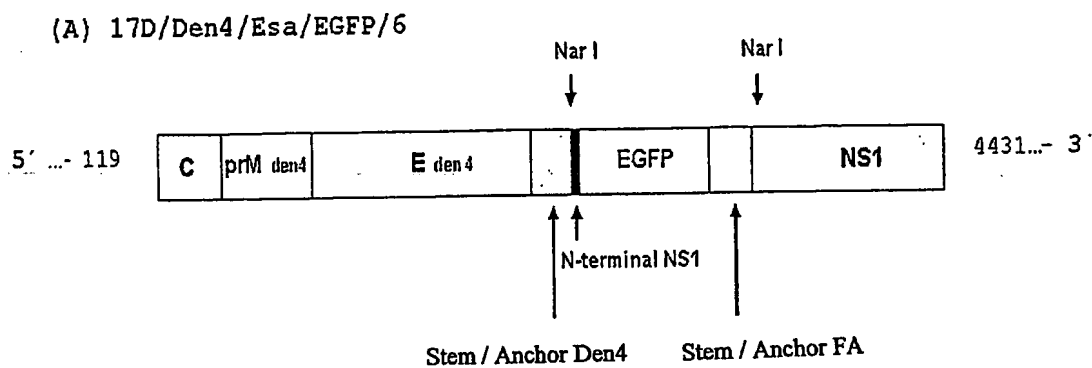

(B) Position of genic sequences in the chimeric viral genome 17D/FA/Den4/Esa/EGFP/6

Gene/position(nt) 17D/FA/Den4/Esa/EGFP/6	5'	3'
C	119	484
prM den4	485	982
E den4	983	2467
E – stem anchor domain of E den4	2180	2467
N– terminal of NS1	2468	2494
EGFP	2495	3208
EGFP – stem anchor domain of E	3209	3496
NS1	3497	4552

Figure 20
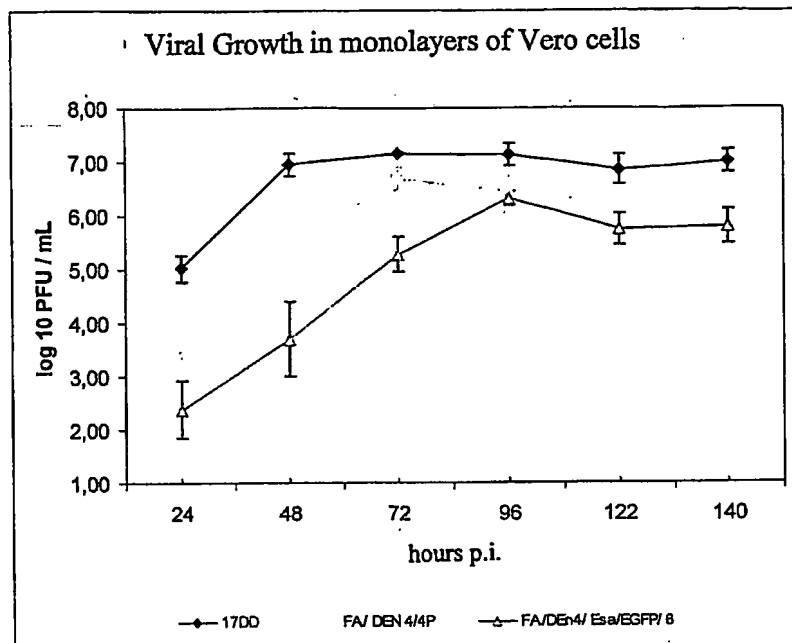
Figure 21

virus	hours p.i.	24	48	72	96	122	140
17DD		5,02 ± 0,25	6,95 ± 0,21	7,17 ± 0,03	7,13 ± 0,21	6,84 ± 0,28	7,01 ± 0,21
FA/ DEN 4/4P	log10 PFU/mL	3,53 ± 0,27	5,35 ± 0,12	6,69 ± 0,21	6,45 ± 0,40	6,21 ± 0,28	6,42 ± 0,18
FA/DEn4/ Esa/EGFP/ 6		2,38 ± 0,54	3,70 ± 0,69	5,27 ± 0,33	6,31 ± 0,13	5,74 ± 0,28	5,79 ± 0,31

Genetic stability of 17D/FA/Den4/Esa/EGFP/6 virus in serial culture in monolayers of Vero cells

Figure 22

Central region of VFA genome

Figure 23

Figure 24

TSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA

Figure 25

Kinetic of viral growth in monolayers of Vero cells

Figure 26
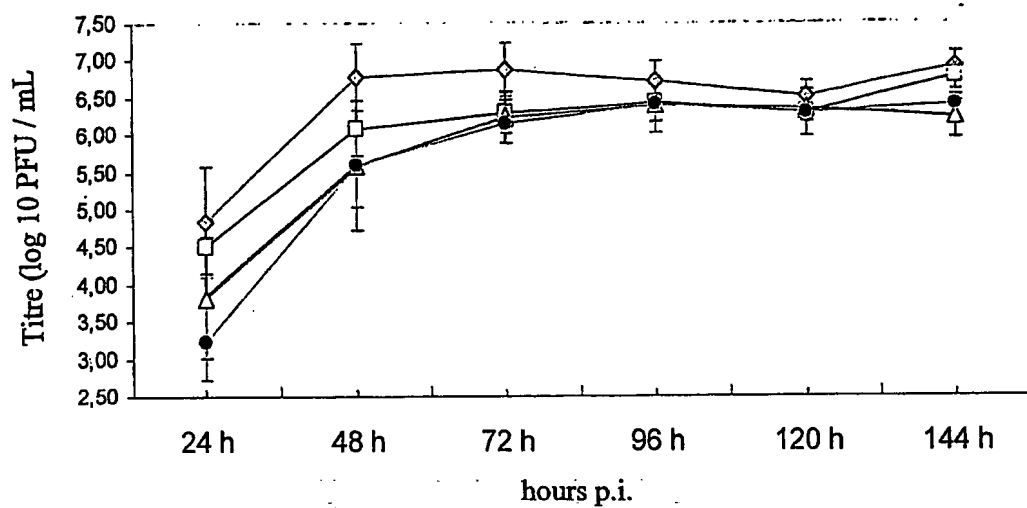
virus	Hours (p.i.)	24 h	48 h	72 h	96 h	120 h	144 h
17DD	log10 PFU / mL	4,84 ± 0,74	6,78 ± 0,45	6,88 ± 0,36	6,71 ± 0,28	6,51 ± 0,21	6,91 ± 0,22
17D/E200T3		4,52 ± 0,38	6,10 ± 0,37	6,29 ± 0,18	6,43 ± 0,25	6,29 ± 0,32	6,79 ± 0,33
17D/Esatrun/ 4 glic		3,83 ± 0,81	5,60 ± 0,86	6,25 ± 0,35	6,41 ± 0,05	6,36 ± 0,18	6,24 ± 0,29
17D/Esa/ 5.1 glic		3,23 ± 0,51	5,61 ± 0,58	6,16 ± 0,12	6,41 ± 0,37	6,29 ± 0,02	6,40 ± 0,19

SEQUENCE LISTING

1) GENERAL INFORMATION:

I.a) Applicant: Fundação Oswaldo Cruz

I.b) Address: Av. Brasil, 4365, Manguinhos – 21040-900 –
Rio de Janeiro – RJ

II) Title of the Invention: "Method for the production of
recombinant virus, DNA constructs, recombinant virus and
vaccine composition."

III) Number of Sequence: 30 (thirty)

IV) Computer readable form:

IV.a) Médium type: Floppy disk

IV.b) Computer: IBM PC compatible.

IV.c) Operating system: PC-DOS/MS-DOS.

2) GENERAL INFORMATION FOR SEQUENCES:

I.a) Identifier Number for Sequence: SEQ ID No. 1

II) Sequence Characteristics:

II.a) Length: 27

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) position in the map: 5' Gene NS1

　　　　　　GATCAAGGATGCGCCATCAACTTTGGC

I.a) Identifier Number for Sequence: SEQ ID No. 2

II) Sequence Characteristics:

II.a) Length: 718

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome: EGFP, genic sequence

III.a) position in the map:

GTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGG

CGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACG

GCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC

CTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAA

GCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCT

TCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACC

CTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGG

GCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGA

AGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAG

CTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGA

CAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATC

ACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTG

TACAAG

I.a) Identifier Number for Sequence: SEQ ID No. 3

II) Sequence Characteristics:

II.a) Length: 288

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) position in the map: rod and anchor of the protein E
gene

AAGTTGTTCACTCAGACCATGAAAGGCGTGGAACGCCTGGCCGTCATGGGAGACACCGC

CTGGGATTTCAGCTCCGCTGGAGGGTTCTTCACTTCGGTTGGGAAAGGAATTCATACGG

TGTTTGGCTCTGCCTTTCAGGGGCTATTTGGCGGCTTGAACTGGATAACAAAGGTCATC

ATGGGGGCGGTACTTATATGGGTTGGCATCAACACAAGAAACATGACAATGTCCATGAG

CATGATCTTGGTAGGAGTGATCATGATGTTTTTGTCTCTAGGAGTTGGCGCC

I.a) Identifier Number for sequence: SEQ ID No. 4

II) Sequence Characteristics:

II.a) Length: 1.029

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) position in the map: 5' terminal NS1- EGFP- rod and
anchor of the protein E gene

GATCAAGGATGCGCCATCAACTTTGGCGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGT

GGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCG

GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACC

GGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTG

CTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCG

AAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGC

GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGA

CTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACA

ACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGC

CACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCAT

CGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGA

GCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCC

GGGATCACTCTCGGCATGGACGAGCTGTACAAGAAGTTGTTCACTCAGACCATGAAAGG

CGTGGAACGCCTGGCCGTCATGGGAGACACCGCCTGGGATTTCAGCTCCGCTGGAGGGT

TCTTCACTTCGGTTGGGAAAGGAATTCATACGGTGTTTGGCTCTGCCTTTCAGGGGCTA

TTTGGCGGCTTGAACTGGATAACAAAGGTCATCATGGGGGCGGTACTTATATGGGTTGG

CATCAACACAAGAAACATGACAATGTCCATGAGCATGATCTTGGTAGGAGTGATCATGA

TGTTTTTGTCTCTAGGAGTTGGCGCC

I.a) Identifier Number for sequence: SEQ ID No. 5

II) Sequence Characteristics:

II.a) Length: 9

II.b) Type: Protein

III) Position in the genome:

III.a) position in the map: N-terminal NS1

DQGCAINFG

I.a) Identifier Number for Sequence: SEQ ID No. 6

II) Sequence Characteristics:

II.a) Length: 242

II.b) Type: Protein

III) Position in the genome:

III.a) Position in the map: EGFP

EGFP

VSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPT

LVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDT

LVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQ

LADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDEL

YK

I.a) Identifier Number for Sequence: SEQ ID No. 7

II) Sequence Characteristics:

II.a) Length: 96

II.b) Type: Protein

III) Position in the genome:

III.a) Position in the map: rod and anchor of protein E

KLFTQTMKGVERLAVMGDTAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVI

MGAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA

I.a) Identifier Number for Sequence: SEQ ID No. 8

II) Sequence Characteristics:

II.a) Length: 343

II.b) Type: exogenous protein expressed in the recombinant
virus consisting of N-terminal from NS1 fused to EGFP
protein followed by the rod and anchor domains of the

protein E

III) Position in the genome:

III.a) Position in the map: N-terminal NS1- EGFP - rod and anchor of protein E

DQGCAINFGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT
GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTR
AEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIR
HNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA
GITLGMDELYKKLFTQTMKGVERLAVMGDTAWDFSSAGGFFTSVGKGIHTVFGSAFQGL
FGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVGA

I.a) Identifier Number for Sequence: SEQ ID No. 9

II) Sequence Characteristics:

II.a) Length: 62

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 328

5´CTAGGAGTTGGCGCCGATCAAGGATGCGCCATCAACTTTGGCGTGAGCAAGGGCGAG

GAGCT 3

I.a) Identifier Number for Sequence: SEQ ID No. 10

II) Sequence Characteristics:

II.a) Length: 51

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG
329

5´GCCTTTCATGGTCTGAGTGAACAACTTCTTGTACAGCTCGTCCATGCCGAG 3´

I.a) Identifier Number for Sequence: SEQ ID No. 11

II) Sequence Characteristics:

II.a) Length: 51

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG
330

5´CTCGGCATGGACGAGCTGTACAAGAAGTTGTTCACTCAGACCATGAAAGGC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 12

II) Sequence Characteristics:

II.a) Length: 46

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 331

5´GCCAAAGTTGATGGCGCATCCTTGATCGGCGCCAACTCCTAGAGAC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 13

II) Sequence Characteristics:

II.a) Length: 11.890

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: nucleotide sequence of the genome of the 17D/Esa/5.1$_{g1ic}$ recombinant virus, positive polarity.

5'

AGTAAATCCTGTGTGCTAATTGAGGTGCATTGGTCTGCAAATCGAGTTGCTAGGCAATAAACACATTTGG

ATTAATTTTAATCGTTCGTTGAGCGATTAGCAGAGAACTGACCAGAACATGTCTGGTCGTAAAGCTCAGG

GAAAAACCCTGGGCGTCAATATGGTACGACGAGGAGTTCGCTCCTTGTCAAACAAAATAAAACAAAAAAC

9

AAAACAAATTGGAAACAGACCTGGACCTTCAAGAGGTGTTCAAGGATTTATCTTTTTCTTTTTGTTCAAC

ATTTTGACTGGAAAAAAGATCACAGCCCACCTAAAGAGGTTGTGGAAAATGCTGGACCCAAGACAAGGCT

TGGCTGTTCTAAGGAAAGTCAAGAGAGTGGTGGCCAGTTTGATGAGAGGATTGTCCTCAAGGAAACGCCG

TTCCCATGATGTTCTGACTGTGCAATTCCTAATTTTGGGAATGCTGTTGATGACGGGTGGAGTGACCTTG

GTGCGGAAAAACAGATGGTTGCTCCTAAATGTGACATCTGAGGACCTCGGGAAAACATTCTCTGTGGGCA

CAGGCAACTGCACAACAAACATTTTGGAAGCCAAGTACTGGTGCCCAGACTCAATGGAATACAACTGTCC

CAATCTCAGTCCAAGAGAGGAGCCAGATGACATTGATTGCTGGTGCTATGGGGTGGAAAACGTTAGAGTC

GCATATGGTAAGTGTGACTCAGCAGGCAGGTCTAGGAGGTCAAGAAGGGCCATTGACTTGCCTACGCATG

AAAACCATGGTTTGAAGACCCGGCAAGAAAAATGGATGACTGGAAGAATGGGTGAAAGGCAACTCCAAAA

GATTGAGAGATGGTTCGTGAGGAACCCCTTTTTTGCAGTGACGGCTCTGACCATTGCCTACCTTGTGGGA

AGCAACATGACGCAACGAGTCGTGATTGCCCTACTGGTCTTGGCTGTTGGTCCGGCCTACTCAGCTCACT

GCATTGGAATTACTGACAGGGATTTCATTGAGGGGGTGCATGGAGGAACTTGGGTTTCAGCTACCCTGGA

GCAAGACAAGTGTGTCACTGTTATGGCCCCTGACAAGCCTTCATTGGACATCTCACTAGAGACAGTAGCC

ATTGATAGACCTGCTGAGGTGAGGAAAGTGTGTTACAATGCAGTTCTCACTCATGTGAAGATTAATGACA

AGTGCCCCAGCACTGGAGAGGCCCACCTAGCTGAAGAGAACGAAGGGGACAATGCGTGCAAGCGCACTTA

TTCTGATAGAGGCTGGGGCAATGGCTGTGGCCTATTTGGGAAAGGGAGCATTGTGGCATGCGCCAAATTC

ACTTGTGCCAAATCCATGAGTTTGTTTGAGGTTGATCAGACCAAAATTCAGTATGTCATCAGAGCACAAT

TGCATGTAGGGGCCAAGCAGGAAAATTGGAATACCAGCATTAAGACTCTCAAGTTTGATGCCCTGTCAGG

CTCCCAGGAAGTCGAGTTCATTGGGTATGGAAAAGCTACACTGGAATGCCAGGTGCAAACTGCGGTGGAC

TTTGGTAACAGTTACATCGCAGAGATGGATATCGAGAGCTGGATAGTGGACAGACAGTGGGCCCAGGACT

TGACCCTGCCATGGCAGAGTGGAAGTGGCGGGGTGTGGAGAGAGATGCATCATCTTGTCGAATTTGAACC

TCCGCATGCCGCCACTATCAGAGTACTGGCCCTGGGAAACCAGGAAGGCTCCTTGAAAACAGCTCTTACT

GGCGCAATGAGGGTTACAAAGGACACAAATGACAACAACCTTTACAAACTACATGGTGGACATGTTTCTT

GCAGAGTGAAATTGTCAGCTTTGACACTCAAGGGGACATCCTACAAAATATGCACTGACAAAATGTTTTT

TGTCAAGAACCCAACTGACACTGGCCATGGCACTGTTGTGATGCAGGTGAAAGTGTCAAAAGGAACCCCC

TGCAGGATTCCAGTGATAGTAGCTGATGATCTTACAGCGGCAATCAATAAAGGCATTTTGGTTACAGTTA

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TTCACTCAGACCATGAAAGGCGTGGAACGCCTGGCCGTCATGGGAGACACCGCCTGGGATTTCAGCTCCG

CTGGAGGGGTTCTTCACTTCGGTTGGGAAAGGAATTCATACGGTGTTTGGCTCTGCCTTTCAGGGGCTATT

TGGCGGCTTGAACTGGATAACAAAGGTCATCATGGGGGCGGTACTCATATGGGTTGGCATCAACACAAGA

AACATGACAATGTCCATGAGCATGATCTTGGTAGGAGTGATCATGATGTTTTTGTCTCTAGGAGTTGGCG

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CGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAG

CTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACC

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GACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGAAGTTGTTCACTCAGACCATGAAAGGC

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AAAGGTCATCATGGGGGCGGTACTTATATGGGTTGGCATCAACACAAGAAACATGACAATGTCCATGAGC

ATGATCTTGGTAGGAGTGATCATGATGTTTTTGTCTCTAGGAGTTGGCGCCGATCAAGGATGCGCCATCA

ACTTTGGCAAGAGAGAGCTCAAGTGCGGAGATGGTATCTTCATATTTAGAGACTCTGATGACTGGCTGAA

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CGTGTCCGCCATATCCCAGACTGAGGTGAAGGAAGAAGGAAAGGAGGAGCTCCAAGAGATCCCGACAATG

CTAAAGAAAGGAATGACAACTGTCCTTGATTTTCATCCTGGAGCTGGGAAGACAAGACGTTTCCTCCCAC

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TGAAATGAAGGAGGCTTTTCACGGCCTGGACGTGAAATTCCACACACAGGCTTTTTCCGCTCACGGCAGC

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TTAACTGGGAAGTGATCATTATGGATGAAGCCCATTTTTTGGATCCAGCTAGCATAGCCGCTAGAGGTTG

GGCAGCGCACAGAGCTAGGGCAAATGAAAGTGCAACAATCTTGATGACAGCCACACCGCCTGGGACTAGT

GATGAATTTCCACATTCAAATGGTGAAATAGAAGATGTTCAAACGGACATACCCAGTGAGCCCTGGAACA

CAGGGCATGACTGGATCCTGGCTGACAAAAGGCCCACGGCATGGTTCCTTCCATCCATCAGAGCTGCAAA

TGTCATGGCTGCCTCTTTGCGTAAGGCTGGAAAGAGTGTGGTGGTCCTGAACAGGAAAACCTTTGAGAGA

GAATACCCCACGATAAAGCAGAAGAAACCTGACTTTATATTGGCCACTGACATAGCTGAAATGGGAGCCA

ACCTTTGCGTGGAGCGAGTGCTGGATTGCAGGACGGCTTTTAAGCCTGTGCTTGTGGATGAAGGGAGGAA

GGTGGCAATAAAAGGGCCACTTCGTATCTCCGCATCCTCTGCTGCTCAAAGGAGGGGGCGCATTGGGAGA

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CAGCCAACGAGCTAGGCATGCTGGAGAAAACCAAAGAGGACCTCTTTGGGAAGAAGAACTTAATTCCATC

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GCCATGCTCCACTGGTCTCTCATTTTACCTGGAATCAAAGCGCAGCAGTCAAAGCTTGCACAGAGAAGGG

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ACTCATGAAATGTACTACGTGTCTGGAGCCCGCAGCAATGTCACATTTACTGTGAACCAAACATCCCGCC

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CCGTGCCATATGGTATATGTGGCTGGGAGCGCGGTATCTTGAGTTTGAGGCCCTGGGATTCCTGAATGAG

GACCATTGGGCTTCCAGGGAAAACTCAGGAGGAGGAGTGGAAGGCATTGGCTTACAATACCTAGGATATG

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TACAAACCACGGGTGGAGAACCGGACTCCCCACAACCTGAAACCGGGATATAAACCACGGCTGGAGAACC

GGGCTCCGCACTTAAAATGAAACAGAAACCGGGATAAAAACTACGGATGGAGAACCGGACTCCACACATT

GAGACAGAAGAAGTTGTCAGCCCAGAACCCCACACGAGTTTTGCCACTGCTAAGCTGTGAGGCAGTGCAG

GCTGGGACAGCCGACCTCCAGGTTGCGAAAAACCTGGTTTCTGGGACCTCCCACCCCAGAGTAAAAAGAA

CGGAGCCTCCGCTACCACCCTCCCACGTGGTGGTAGAAAGACGGGGTCTAGAGGTTAGAGGAGACCCTCC

AGGGAACAAATAGTGGGACCATATTGACGCCAGGGAAAGACCGGAGTGGTTCTCTGCTTTTCCTCCAGAG

GTCTGTGAGCACAGTTTGCTCAAGAATAAGCAGACCTTTGGATGACAAACACAAAACCAC 3'

I.a) Identifier Number for Sequence: SEQ ID No. 14

II) Sequence Characteristics:

II.a) Length: 3754 aminoacid residues

II.b) Type: aminoacids

III) Position in the genome:

III.a) Position in the map: polyprotein sequence from the
17D/Esa/5.1$_{glic}$ recombinant virus.

MSGRKAQGKTLGVNMVRRGVRSLSNKIKQKTKQIGNRPGPSRGVQGFIFFFLFNILTGKKITAHLKRLWK

MLDPRQGLAVLRKVKRVVASLMRGLSSRKRRSHDVLTVQFLILGMLLMTGGVTLVRKNRWLLLNVTSEDL

GKTFSVGTGNCTTNILEAKYWCPDSMEYNCPNLSPREEPDDIDCWCYGVENVRVAYGKCDSAGRSRRSRR

AIDLPTHENHGLKTRQEKWMTGRMGERQLQKIERWFVRNPFFAVTALTIAYLVGSNMTQRVVIALLVLAV

GPAYSAHCIGITDRDFIEGVHGGTWVSATLEQDKCVTVMAPDKPSLDISLETVAIDRPAEVRKVCYNAVL

THVKINDKCPSTGEAHLAEENEGDNACKRTYSDRGWGNGCGLFGKGSIVACAKFTCAKSMSLFEVDQTKI

QYVIRAQLHVGAKQENWNTSIKTLKFDALSGSQEVEFIGYGKATLECQVQTAVDFGNSYIAEMDIESWIV

DRQWAQDLTLPWQSGSGGVWREMHHLVEFEPPHAATIRVLALGNQEGSLKTALTGAMRVTKDTNDNNLYK

LHGGHVSCRVKLSALTLKGTSYKICTDKMFFVKNPTDTGHGTVVMQVKVSKGTPCRIPVIVADDLTAAIN

KGILVTVNPIASTNDDEVLIEVNPPFGDSYIIVGRGDSRLTYQWHKEGSSIGKLFTQTMKGVERLAVMGD

TAWDFSSAGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMM

FLSLGVGADQGCAINFGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKL

PVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRI

ELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQNTPIGDGPV

LLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYKKLFTQTMKGVERLAVMGDTAWDFSS

AGGFFTSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMMFLSLGVG

ADQGCAINFGKRELKCGDGIFIFRDSDDWLNKYSYYPEDPVKLASIVKASFEEGKCGLNSVDSLEHEMWR

SRADEINTIFEENEVDISVVVQDPKNVYQRGTHPFSRIRDGLQYGWKTWGKNLVFSPGRKNGSFIIDGKS

RKECPFSNRVWNSFQIEEFGTGVFTTRVYMDAVFEYTIDCDGSILGAAVNGKKSAHGSPTFWMGSHEVNG

TWMIHTLEALDYKECEWPLTHTIGTSVEESEMFMPRSIGGPVSSHNHIPGYKVQTNGPWMQVPLEVKREA

CPGTSVIIDGNCDGRGKSTRSTTDSGKVIPEWCCRSCTMPPVSFHGSDGCWYPMEIRPRKTHESHLVRSW

VTAGEIHAVPFGLVSMMIAMEVVLRKRQGPKQMLVGGVVLLGAMLVGQVTLLDLLKLTVAVGLHFHEMNN

GGDAMYMALIAAFSIRPGLLIGFGLRTLWSPRERLVLTLGAAMVEIALGGVMGGLWKYLNAVSLCILTIN

AVASRKASNTILPLMALLTPVTMAEVRLAAMFFCAMVIIGVLHQNFKDTSMQKTIPLVALTLTSYLGLTQ

PFLGLCAFLATRIFGRRSIPVNEALAAAGLVGVLAGLAFQEMENFLGPIAVGGLLMMLVSVAGRVDGLEL

KKLGEVSWEEEAEISGSSARYDVALSEQGEFKLLSEEKVPWDQVVMTSLALVGAALHPFALLLVLAGWLF

HVRGARRSGDVLWDIPTPKIIEECEHLEDGIYGIFQSTFLGASQRGVGVAQGGVFHTMWHVTRGAFLVRN

GKKLIPSWASVKEDLVAYGGSWKLEGRWDGEEEVQLIAAVPGKNVVNVQTKPSLFKVRNGGEIGAVALDY

PSGTSGSPIVNRNGEVIGLYGNGILVGDNSFVSAISQTEVKEEGKEELQEIPTMLKKGMTTVLDFHPGAG

KTRRFLPQILAECARRRLRTLVLAPTRVVLSEMKEAFHGLDVKFHTQAFSAHGSGREVIDAMCHATLTYR

MLEPTRVVNWEVIIMDEAHFLDPASIAARGWAAHRARANESATILMTATPPGTSDEFPHSNGEIEDVQTD

IPSEPWNTGHDWILADKRPTAWFLPSIRAANVMAASLRKAGKSVVVLNRKTFEREYPTIKQKKPDFILAT

DIAEMGANLCVERVLDCRTAFKPVLVDEGRKVAIKGPLRISASSAAQRRGRIGRNPNRDGDSYYYSEPTS

ENNAHHVCWLEASMLLDNMEVRGGMVAPLYGVEGTKTPVSPGEMRLRDDQRKVFRELVRNCDLPVWLSWQ

VAKAGLKTNDRKWCFEGPEEHEILNDSGETVKCRAPGGAKKPLRPRWCDERVSSDQSALSEFIKFAEGRR

GAAEVLVVLSELPDFLAKKGGEAMDTISVFLHSEEGSRAYRNALSMMPEAMTIVMLFILAGLLTSGMVIF

FMSPKGISRMSMAMGTMAGCGYLMFLGGVKPTHISYVMLIFFVLMVVVIPEPGQQRSIQDNQVAYLIIGI

LTLVSAVAANELGMLEKTKEDLFGKKNLIPSSASPWSWPDLDLKPGAAWTVYVGIVTMLSPMLHHWIKVE

YGNLSLSGIAQSASVLSFMDKGIPFMKMNISVIMLLVSGWNSITVMPLLCGIGCAMLHWSLILPGIKAQQ

SKLAQRRVFHGVAKNPVVDGNPTVDIEEAPEMPALYEKKLALYLLLALSLASVAMCRTPFSLAEGIVLAS

AALGPLIEGNTSLLWNGPMAVSMTGVMRGNHYAFVGVMYNLWKMKTGRRGSANGKTLGEVWKRELNLLDK

RQFELYKRTDIVEVDRDTARRHLAEGKVDTGVAVSRGTAKLRWFHERGYVKLEGRVIDLGCGRGGWCYYA

AAQKEVSGVKGFTLGRDGHEKPMNVQSLGWNIITFKDKTDIHRLEPVKCDTLLCDIGESSSSSVTEGERT

VRVLDTVEKWLACGVDNFCVKVLAPYMPDVLEKLELLQRRFGGTVIRNPLSRNSTHEMYYVSGARSNVTF

TVNQTSRLLMRRMRRPTGKVTLEADVILPIGTRSVETDKGPLDKEAIEERVERIKSEYMTSWFYDNDNPY

RTWHYCGSYVTKTSGSAASMVNGVIKILTYPWDRIEEVTRMAMTDTTPFGQQRVFKEKVDTRAKDPPAGT

RKIMKVVNRWLFRHLAREKSPRLCTKEEFIAKVRSHAAIGAYLEEQEQWKTANEAVQDPKFWELVDEERK

LHQQGRCRTCVYNMMGKREKKLSEFGKAKGSRAIWYMWLGARYLEFEALGFLNEDHWASRENSGGGVEGI

GLQYLGYVIRDLAAMDGGGFYADDTAGWDTRITEADLDDEQEILNYMSPHHKKLAQAVMEMTYKNKVVKV

LRPAPGGKAYMDVISRRDQRGSGQVVTYALNTITNLKVQLIRMAEAEMVIHHQHVQDCDESVLTRLEAWL

TEHGCNRLKRMAVSGDDCVVRPIDDRFGLALSHLNAMSKVRKDISEWQPSKGWNDWENVPFCSHHFHELQ

LKDGRRIVVPCREQDELIGRGRVSPGNGWMIKETACLSKAYANMWSLMYFHKRDMRLLSLAVSSAVPTSW

VPQGRTTWSIHGKGEWMTTEDMLEVWNRVWITNNPHMQDKTMVKKWRDVPYLTKRQDKLCGSLIGMTNRA

TWASHIHLVIHRIRTLIGQEKYTDYLTVMDRYSVDADLQLGELI

I.a) Identifier Number for Sequence: SEQ ID No. 15

II) Sequence Characteristics:

II.a) Length: 10.861 nucleotides

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: nucleotide sequence of the
genome of the YF17D virus, named YF17D/Eglic.

AGTAAATCCTGTGTGCTAATTGAGGTGCATTGGTCTGCAAATCGAGTTGCTAGGCAATAAACACATTTGG

ATTAATTTTAATCGTTCGTTGAGCGATTAGCAGAGAACTGACCAGAACATGTCTGGTCGTAAAGCTCAGG

GAAAAACCCTGGGCGTCAATATGGTACGACGAGGAGTTCGCTCCTTGTCAAACAAAATAAAACAAAAAAC

AAAACAAATTGGAAACAGACCTGGACCTTCAAGAGGTGTTCAAGGATTTATCTTTTTCTTTTTGTTCAAC

ATTTTGACTGGAAAAAAGATCACAGCCCACCTAAAGAGGTTGTGGAAAATGCTGGACCCAAGACAAGGCT

TGGCTGTTCTAAGGAAAGTCAAGAGAGTGGTGGCCAGTTTGATGAGAGGATTGTCCTCAAGGAAACGCCG

TTCCCATGATGTTCTGACTGTGCAATTCCTAATTTTGGGAATGCTGTTGATGACGGGTGGAGTGACCTTG

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AGCAACATGACGCAACGAGTCGTGATTGCCCTACTGGTCTTGGCTGTTGGTCCGGCCTACTCAGCTCACT

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ACTTGTGCCAAATCCATGAGTTTGTTTGAGGTTGATCAGACCAAAATTCAGTATGTCATCAGAGCACAAT

TGCATGTAGGGGCCAAGCAGGAAAATTGGAATACCAGCATTAAGACTCTCAAGTTTGATGCCCTGTCAGG

CTCCCAGGAAGTCGAGTTCATTGGGTATGGAAAAGCTACACTGGAATGCCAGGTGCAAACTGCGGTGGAC

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TGACCCTGCCATGGCAGAGTGGAAGTGGCGGGGTGTGGAGAGAGATGCATCATCTTGTCGAATTTGAACC

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GCAGAGTGAAATTGTCAGCTTTGACACTCAAGGGGACATCCTACAAAATATGCACTGACAAAATGTTTTT

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AGAGGTTAGAGGAGACCCTCCAGGGAACAAATAGTGGGACCATATTGACGCCAGGGAAAGACCGGAGTGG

TTCTCTGCTTTTCCTCCAGAGGTCTGTGAGCACAGTTTGCTCAAGAATAAGCAGACCTTTGGATGACAAA

CACAAAACCAC

I.a) Identifier Number for Sequence: SEQ ID No. 16

II) Sequence Characteristics:

II.a) Length: 21

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG

174, sense.

5´ CGGGGTGTGGAGAGAGATGCA 3´

I.a) Identifier Number for Sequence: SEQ ID No. 17

II) Sequence Characteristics:

II.a) Length: 21

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG

19, reverse

5´ GGGAGTCAACTGAATTTAGGC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 18

II) Sequence Characteristics:

II.a) Length: 47   :

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 295, sense

5´GCTTGATTCCCACCGGTATGGCGTTTTCCCTCAGCACAAGAGATGGC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 19

II) Sequence Characteristics:

II.a) Length: 19

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 296, reverse

5´ GGGCAGAATGCATGGCTCC 3'

I.a) Identifier Number for Sequence: SEQ ID No. 20

II) Sequence Characteristics:

II.a) Length: 19

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 297, sense

5' GGAGCCATGCATTCTGCCC 3'

I.a) Identifier Number for Sequence: SEQ ID No. 21

II) Sequence Characteristics:

II.a) Length: 49

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 298, reverse

5' GACGCCACACAACCCATGTCGGCGCCAACTGTGAAGCCCAGAAACAGAG 3´

I.a) Identifier Number for Sequence: SEQ ID No. 22

II) Sequence Characteristics:

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: circular

III) Position in the genome:

III.a) Position in the map: plasmid derived from pACNR1180

26

containing cDNA viral of the Den4/FA/EGFP chimeric virus
(14.498 nucleotides)

5´GTGACCACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAAC

GTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCAC

TCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACA

GGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCT

TCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGA

ATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGAC

GTCGATCGCGGCCGCTAGCGATGACCCTGCTGATTGGTTCGCTGACCATTTCCGGGTGCGGGAC

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AGTAAATCCTGTGTGCTAATTGAGGTGCATTGGTCTGCAAATCGAGTTGCTAGGCAATAAACAC

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CAAAATAAAACAAAAAACAAAACAAATTGGAAACAGACCTGGACCTTCAAGAGGTGTTCAAGGA

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TGTGGAAAATGCTGGACCCAAGACAAGGCTTGGCTGTTCTAAGGAAAGTCAAGAGAGTGGTGGC

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CCCTCATGATAGTGGCAAAACATGAAAGGGGGAGACCTCTCTTGTTTAAGACAACAGAGGGGAT

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TATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAAC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 23

II) Sequence Characteristics:

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: nucleotide sequence of the

genome of the Den4/FA/Esa/EGFP virus (11.905 nucleotides)

5´

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CTAGGCATGCTGGAGAAAACCAAAGAGGACCTCTTTGGGAAGAAGAACTTAATTCCATCTAGTG

CTTCACCCTGGAGTTGGCCGGATCTTGACCTGAAGCCAGGAGCTGCCTGGACAGTGTACGTTGG

CATTGTTACAATGCTCTCTCCAATGTTGCACCACTGGATCAAAGTCGAATATGGCAACCTGTCT

CTGTCTGGAATAGCCCAGTCAGCCTCAGTCCTTTCTTTCATGGACAAGGGGATACCATTCATGA

AGATGAATATCTCGGTCATAATGCTGCTGGTCAGTGGCTGGAATTCAATAACAGTGATGCCTCT

GCTCTGTGGCATAGGGTGCGCCATGCTCCACTGGTCTCTCATTTTACCTGGAATCAAAGCGCAG

CAGTCAAAGCTTGCACAGAGAAGGGTGTTCCATGGCGTTGCCAAGAACCCTGTGGTTGATGGGA

ATCCAACAGTTGACATTGAGGAAGCTCCTGAAATGCCTGCCCTTTATGAGAAGAAACTGGCTCT

ATATCTCCTTCTTGCTCTCAGCCTAGCTTCTGTTGCCATGTGCAGAACGCCCTTTTCATTGGCT

GAAGGCATTGTCCTAGCATCAGCTGCCTTAGGGCCGCTCATAGAGGGAAACACCAGCCTTCTTT

GGAATGGACCCATGGCTGTCTCCATGACAGGAGTCATGAGGGGGAATCACTATGCTTTTGTGGG

AGTCATGTACAATCTATGGAAGATGAAAACTGGACGCCGGGGGAGCGCGAATGGAAAAACTTTG

GGTGAAGTCTGGAAGAGGGAACTGAATCTGTTGGACAAGCGACAGTTTGAGTTGTATAAAAGGA

CCGACATTGTGGAGGTGGATCGTGATACGGCACGCAGGCATTTGGCCGAAGGGAAGGTGGACAC

CGGGGTGGCGGTCTCCAGGGGGACCGCAAAGTTAAGGTGGTTCCATGAGCGTGGCTATGTCAAG

CTGGAAGGTAGGGTGATTGACCTGGGGTGTGGCCGCGGAGGCTGGTGTTACTACGCTGCTGCGC

AAAAGGAAGTGAGTGGGGTCAAAGGATTTACTCTTGGAAGAGACGGCCATGAGAAACCCATGAA

TGTGCAAAGTCTGGGATGGAACATCATCACCTTCAAGGACAAAACTGATATCCACCGCCTAGAA

CCAGTGAAATGTGACACCCTTTTGTGTGACATTGGAGAGTCATCATCGTCATCGGTCACAGAGG

GGGAAAGGACCGTGAGAGTTCTTGATACTGTAGAAAAATGGCTGGCTTGTGGGGTTGACAACTT

CTGTGTGAAGGTGTTAGCTCCATACATGCCAGATGTTCTTGAGAAACTGGAATTGCTCCAAAGG

AGGTTTGGCGGAACAGTGATCAGGAACCCTCTCTCCAGGAATTCCACTCATGAAATGTACTACG

TGTCTGGAGCCCGCAGCAATGTCACATTTACTGTGAACCAAACATCCCGCCTCCTGATGAGGAG

AATGAGGCGTCCAACTGGAAAAGTGACCCTGGAGGCTGACGTCATCCTCCCAATTGGGACACGC

AGTGTTGAGACAGACAAGGGACCCCTGGACAAAGAGGCCATAGAAGAAAGGGTTGAGAGGATAA

AATCTGAGTACATGACCTCTTGGTTTTATGACAATGACAACCCCTACAGGACCTGGCACTACTG

TGGCTCCTATGTCACAAAAACCTCAGGAAGTGCGGCGAGCATGGTAAATGGTGTTATTAAAATT

CTGACATATCCATGGGACAGGATAGAGGAGGTCACCAGAATGGCAATGACTGACACAACCCCTT

TTGGACAGCAAAGAGTGTTTAAAGAAAAAGTTGACACCAGAGCAAAGGATCCACCAGCGGGAAC

TAGGAAGATCATGAAAGTTGTCAACAGGTGGCTGTTCCGCCACCTGGCCAGAGAAAAGAGCCCC

AGACTGTGCACAAAGGAAGAATTTATTGCAAAAGTCCGAAGTCATGCAGCCATTGGAGCTTACC

TGGAAGAACAAGAACAGTGGAAGACTGCCAATGAGGCTGTCCAAGACCCAAAGTTCTGGGAACT

GGTGGATGAAGAAAGGAAGCTGCACCAACAAGGCAGGTGTCGGACTTGTGTGTACAACATGATG

GGGAAAAGAGAGAAGAAGCTGTCAGAGTTTGGGAAAGCAAAGGGAAGCCGTGCCATATGGTATA

TGTGGCTGGGAGCGCGGTATCTTGAGTTTGAGGCCCTGGGATTCCTGAATGAGGACCATTGGGC

TTCCAGGGAAAACTCAGGAGGAGGAGTGGAAGGCATTGGCTTACAATACCTAGGATATGTGATC

AGAGACCTGGCTGCAATGGATGGTGGTGGATTCTACGCGGATGACACCGCTGGATGGGACACGC

GCATCACAGAGGCAGACCTTGATGATGAACAGGAGATCTTGAACTACATGAGCCCACATCACAA

AAAACTGGCACAAGCAGTGATGGAAATGACATACAAGAACAAAGTGGTGAAAGTGTTGAGACCA

GCCCCAGGAGGGAAAGCCTACATGGATGTCATAGGTCGACGAGACCAGAGAGGATCCGGGCAGG

TAGTGACTTATGCTCTGAACACCATCACCAACTTGAAAGTCCAATTGATCAGAATGGCAGAAGC

AGAGATGGTGATACATCACCAACATGTTCAAGATTGTGATGAATCAGTTCTGACCAGGCTGGAG

GCATGGCTCACTGAGCACGGATGTAACAGACTGAAGAGGATGGCGGTGAGTGGAGACGACTGTG

TGGTCCGGCCCATCGATGACAGGTTCGGCCTGGCCCTGTCCCATCTCAACGCCATGTCCAAGGT

TAGAAAGGACATATCTGAATGGCAGCCATCAAAAGGGTGGAATGATTGGGAGAATGTGCCCTTC

TGTTCCCACCACTTCCATGAACTACAGCTGAAGGATGGCAGGAGGATTGTGGTGCCTTGCCGAG

AACAGGACGAGCTCATTGGGAGAGGAAGGGTGTCTCCAGGAAACGGCTGGATGATCAAGGAAAC

AGCTTGCCTCAGCAAAGCCTATGCCAACATGTGGTCACTGATGTATTTTCACAAAAGGGACATG

AGGCTACTGTCATTGGCTGTTTCCTCAGCTGTTCCCACCTCATGGGTTCCACAAGGACGCACAA

CATGGTCGATTCATGGGAAAGGGGAGTGGATGACCACGGAAGACATGCTTGAGGTGTGGAACAG

AGTATGGATAACCAACAACCCACACATGCAGGACAAGACAATGGTGAAAAAATGGAGAGATGTC

CCTTATCTAACCAAGAGACAAGACAAGCTGTGCGGATCACTGATTGGAATGACCAATAGGGCCA

CCTGGGCCTCCCACATCCATTTAGTCATCCATCGTATCCGAACGCTGATTGGACAGGAGAAATA

CACTGACTACCTAACAGTCATGGACAGGTATTCTGTGGATGCTGACCTGCAACTGGGTGAGCTT

ATCTGAAACACCATCTAACAGGAATAACCGGGATACAAACCACGGGTGGAGAACCGGACTCCCC

ACAACCTGAAACCGGGATATAAACCACGGCTGGAGAACCGGGCTCCGCACTTAAAATGAAACAG

AAACCGGGATAAAAACTACGGATGGAGAACCGGACTCCACACATTGAGACAGAAGAAGTTGTCA

GCCCAGAACCCCACACGAGTTTTGCCACTGCTAAGCTGTGAGGCAGTGCAGGCTGGGACAGCCG

ACCTCCAGGTTGCGAAAAACCTGGTTTCTGGGACCTCCCACCCCAGAGTAAAAAGAACGGAGCC

TCCGCTACCACCCTCCCACGTGGTGGTAGAAAGACGGGGTCTAGAGGTTAGAGGAGACCCTCCA

GGGAACAAATAGTGGGACCATATTGACGCCAGGGAAAGACCGGAGTGGTTCTCTGCTTTTCCTC

CAGAGGTCTGTGAGCACAGTTTGCTCAAGAATAAGCAGACCTTTGGATGACAAACACAAAACCA

C 3´

I.a) Identifier Number for Sequence: SEQ ID No. 24

II) Sequence Characteristics:

II.a) Length: 19

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG

367

5´CATGGACAGCAGGAGCAGA 3´

I.a) Identifier Number for Sequence: SEQ ID No. 25

II) Sequence Characteristics:

II.a) Length: 198

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: truncated region of the rod and

anchor of the protein E gene

5´ACTTCGGTTGGGAAAGGAATTCATACGGTGTTTGGCTCTGCCTTTCAGGGGCTATTT

GGCGGCTTGAACTGGATAACAAAGGTCATCATGGGGGCGGTACTTATATGGGTTGGCAT

CAACACAAGAAACATGACAATGTCCATGAGCATGATCTTGGTAGGAGTGATCATGATGT

TTTTGTCTCTAGGAGTTGGCGCC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 26

II) Sequence Characteristics:

II.a) Length: 66

II.b) Type: Protein

III) Position in the genome:

III.a) Position in the map: truncated domain of the rod and anchor of the protein E

TSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSMSMILVGVIMMF LSLGVGA

I.a) Identifier Number for Sequence: SEQ ID No. 27

II) Sequence Characteristics:

II.a) Length: 50

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

IIIa) Position in the map: synthetic oligonucleotide: RG 332, reverse

5´CCGTATGAATTCCTTTCCCAACCGAAGTCTTGTACAGCTCGTCCATGCCG 3´

I.a) Identifier Number for Sequence: SEQ ID No. 28

II) Sequence Characteristics:

II.a) Length: 50

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: synthetic oligonucleotide: RG 333, sense

5´CGGCATGGACGAGCTGTACAAGACTTCGGTTGGGAAAGGAATTCATACGG 3´

I.a) Identifier Number for Sequence: SEQ ID No. 29

II) Sequence Characteristics:

II.a) Length: 939

II.b) Type: nucleic acid

II.c) Strandedness: single strand

II.d) Topology: linear

III) Position in the genome:

III.a) Position in the map: 5' terminal NS1- EGFP-truncated rod and the anchor of the protein E gene

5´GATCAAGGATGCGCCATCAACTTTGGCGTGAGCAAGGGCGAGGAGCTGTTCACCGGG
GTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTC
CGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCA
CCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAG
TGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCC
CGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCC
GCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATC
GACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCA
CAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCC
GCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCT

GAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCG

CCGGGATCACTCTCGGCATGGACGAGCTGTACAAGACTTCGGTTGGGAAAGGAATTCAT

ACGGTGTTTGGCTCTGCCTTTCAGGGGCTATTTGGCGGCTTGAACTGGATAACAAAGGT

CATCATGGGGGCGGTACTTATATGGGTTGGCATCAACACAAGAAACATGACAATGTCCA

TGAGCATGATCTTGGTAGGAGTGATCATGATGTTTTTGTCTCTAGGAGTTGGCGCC 3´

I.a) Identifier Number for Sequence: SEQ ID No. 30

II) Sequence Characteristics:

II.a) Length: 313

II.b) Type: Protein

III) Position in the genome:

III.a) Position in the map: expression cassette with N-terminal NS1- EGFP- truncated rod and anchor of the protein E gene

DQGCAINFGVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTT

GKLPVPWPTLVTTLTYGVQCFSRYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTR

AEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIMADKQKNGIKVNFKIR

HNIEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAA

GITLGMDELYKTSVGKGIHTVFGSAFQGLFGGLNWITKVIMGAVLIWVGINTRNMTMSM

SMILVGVIMMFLSLGVGA